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APPLICATION NUMBER: 09/353,617

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## PRIORITY DOCUMENT

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## PATENT APPLICATION TRANSMITTAL LETTER

(Large Entity)

Docket No.

1038-868 MIS:jb

TO THE ASSISTANT COMMISSIONER FOR PATENTS

Transmitted herewith for filing under 35 U.S.C. 111 and 37 C.F.R. 1.53 is the patent application of:

Heena M. Loosmore, et al.

For: **MULTI-COMPONENT VACCINE TO PROTECT AGAINST DISEASE CAUSED BY  
HAEMOPHILUS INFLUENZAE AND MORAXELLA CATARRHALIS**

Enclosed are:

- ☐ Certificate of Mailing with Express Mail Mailing Label No.  
☒ Seventeen (17) sheets of drawings.  
☐ A certified copy of a application.  
☒ Declaration ☐ Signed. ☒ Unsigned.  
☐ Power of Attorney  
☐ Information Disclosure Statement  
☐ Preliminary Amendment  
☐ Other:

## CLAIMS AS FILED

For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	29	- 20 =	9	x \$18.00	\$162.00
Indep. Claims	3	- 3 =	0	x \$78.00	\$0.00
Multiple Dependent Claims (check if applicable) <input type="checkbox"/>					\$0.00
BASIC FEE					\$760.00
TOTAL FILING FEE					\$922.00

- ☒ A check in the amount of \$922.00 to cover the filing fee is enclosed.  
☒ The Commissioner is hereby authorized to charge and credit Deposit Account No. as described below. A duplicate copy of this sheet is enclosed.  
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☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: July 14, 1999

*Michael I. Stewart*  
Michael I. Stewart Signature  
(24,973)

cc:

TITLE OF INVENTION

MULTI-COMPONENT VACCINE TO PROTECT  
AGAINST DISEASE CAUSED BY HAEMOPHILUS  
INFLUENZAE AND MORAXELLA CATARRHALIS

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FIELD OF INVENTION

The present invention relates to the field of vaccinology and, in particular, to a multi-component vaccine comprising recombinant proteins from *Haemophilus influenzae* and *Moraxella catarrhalis*.

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BACKGROUND TO THE INVENTION

*Haemophilus influenzae* is the cause of several serious human diseases, such as meningitis, epiglottitis, septicemia and otitis media. There are six serotypes of *H. influenzae*, designated a to f, that are identified by their capsular polysaccharide. *H. influenzae* type b (Hib) was a major cause of bacterial meningitis until the introduction of several Hib conjugate vaccines in the 1980's (ref. 1. Throughout the application, various references are referred to in parenthesis to more fully describe the state of the art to which this invention are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosures of these references are hereby incorporated by reference into the present disclosure). Vaccines based upon *H. influenzae* type b capsular polysaccharide conjugated to diphtheria toxoid (ref. 2), tetanus toxoid (ref. 3, and US Patent No. 4,496,538), or *Neisseria meningitidis* outer membrane protein (ref. 4) have been effective in reducing *H. influenzae* type b-

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induced meningitis. The other serotypes of *H. influenzae* are associated with invasive disease at low frequencies, although there appears to be an increase in the incidence in disease caused by these strains as the incidence of Hib disease declines (refs. 5, 6). Non-encapsulated or non-typeable *H. influenzae* (NTHi) are also responsible for a wide range of human diseases including otitis media, epiglottitis, pneumonia, and tracheobronchitis. The incidence of NTHi-induced disease has not been affected by the introduction of the Hib vaccines (ref. 7).

Otitis media is the most common illness of early childhood, with 60 to 70% of all children, of less than 2 years of age, experiencing between one and three ear infections (ref. 8). Chronic otitis media is responsible for hearing, speech and cognitive impairments in children. *H. influenzae* infections account for about 30% of the cases of acute otitis media and about 60% of chronic otitis media. *M. catarrhalis* infections account for an additional 15 to 20% of acute otitis media. In the United States alone, treatment of otitis media costs between 1 and 2 billion dollars per year for antibiotics and surgical procedures such as tonsillectomies, adenoidectomies and insertion of tympanostomy tubes. It is estimated that an additional \$30 billion is spent per annum on adjunct therapies such as speech therapy and special education classes. *Moraxella* (*Branhamella*) *catarrhalis* is the third most common cause of otitis media and sinusitis in children, responsible for 15 to 20% of disease. It has also been associated with lower respiratory tract disease in children and adults; including pneumonia and chronic bronchitis and more rarely it can cause

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bacteremia and meningitis (refs. 9, 10, 11). There are no vaccines available to protect against *M. catarrhalis* disease. Furthermore, many of the causative organisms of otitis media are becoming resistant to antibiotic treatment. An effective prophylactic vaccine against otitis media is thus highly desirable.

During natural infection, surface-exposed outer membrane proteins that stimulate an antibody response are potentially important targets for bactericidal and/or protective antibodies and therefore potential vaccine candidates. Barenkamp and Bodor (ref. 12) demonstrated that convalescent sera from children suffering from otitis media due to NTHi, contained antibodies to high molecular weight (HMW) proteins. About 70 to 75% of NTHi strains express the HMW proteins and most of these strains contain two gene clusters termed *hmw1ABC* and *hmw2ABC* (refs. 13, 14). The HMWA proteins have been demonstrated to be adhesins mediating attachment to human epithelial cells (ref. 15). Immunization with a mixture of native HMW1A and HMW2A proteins resulted in partial protection in the chinchilla intrabulla challenge model of otitis media (ref. 16).

US Patent No. 5,603,938 (Barenkamp), assigned to St. Louis University and Washington University and the disclosure of which is incorporated herein by reference, describes the cloning, expression and sequencing of the genes encoding the HMW1 and HMW2 proteins from strain 12 of non-typeable *Haemophilus*. The HMW proteins are a family of proteins from non-typeable *Haemophilus* of molecular weight of about 120 to 125 kDa which are found in non-typeable *Haemophilus*

strains. The HMW proteins are absent from encapsulated strains of *Haemophilus*.

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The production of native HMW proteins from *H. influenzae* strains is very low and a method for producing protective recombinant HMW (rHMW) proteins has been described in United States Patent Application No. 09/167,568 filed October 7, 1998, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. A chinchilla nasopharyngeal colonization model has been developed specifically to demonstrate vaccine efficacy of adhesins (ref. 17) and the rHMW proteins are protective in this model as described in the aforementioned United States Patent Application No. 09/167,568. The rHMW1A and rHMW2A proteins were shown to afford equivalent protection to each other and the rHMW1A protein was chosen for further vaccine studies. In this application, rHMW refers to recombinant HMW1A from NTHi strain 12, although the corresponding recombinant HMW1A protein from other NTHi strains and corresponding rHMW2A protein from NTHi strains may be employed. The corresponding naturally-occurring proteins may be employed.

A second family of high molecular weight adhesion proteins has been identified in about 25% of NTHi and in encapsulated *H. influenzae* strains (refs. 18, 19, 20). U.S. Patent No. 5,646,259 (St. Geme, III et al), assigned to St. Louis University and Washington University, and the disclosure of which is incorporated herein by reference, describes the cloning, expression and sequences of genes encoding what are termed therein the HA1 and HA2 proteins, which have limited homology to the HMW1 and HMW2 proteins of USP 5,603,938.

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The NTHi member of this second family is termed *Haemophilus influenzae* adhesin or Hia (HA1) and the homologous protein found in encapsulated strains is termed *Haemophilus influenzae* surface fibril protein or Hsf (HA2). The *hia* gene was originally cloned from an expression library using convalescent sera from an otitis media patient, which indicates that it is an important immunogen during disease. The prototype Hia and Hsf proteins demonstrate about 82% sequence similarity, although the Hsf protein is considerably larger. The proteins are comprised of conserved amino and carboxy termini and several repeat motifs, with Hsf containing more repeat sequences than Hia.

United States Patent Application No. 09/268,347 filed March 16, 1999 (1038-860), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, describes the production of full-length and N-terminally truncated versions of the Hia protein (rHia) in *E. coli*. These recombinant proteins have been demonstrated to protect against bacteremia caused by *H. influenzae* type a and type b organisms, and to confer partial protection against nasopharyngeal colonization by non-typeable *H. influenzae*. In this application, rHia refers to V38 rHia from NTHi strain 11, although other recombinant full-length and N-terminally truncated Hia proteins from other NTHi strains may be employed. Corresponding naturally-occurring proteins also may be employed.

A high molecular weight adhesin identified in *M. catarrhalis*, has been termed 200 kDa and is described in US Patent No. 5,808,024 (Sasaki et al), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, as well as copending

Untied States Application No. 08/621,944 filed March 26, 1996 (WO 96/34960), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. The 200 kDa protein has been identified in 5 96 out of 109 *M. catarrhalis* strains, including 73 out of 74 otitis media-derived strains, and is postulated to be a virulence factor. There is sequence homology between the *M. catarrhalis* 200 kDa protein and the *H. influenzae* Hia and Hsf proteins. In addition, anti- 10 native 200 kDa antibody recognized the rHia protein on an immunoblot, indicating antigenic relatedness, as demonstrated in the aforementioned copending United States Patent Application No. 09/268,347 (1038-860).

There is no suitable animal model for *M.* 15 *catarrhalis* infection and disease, but a bactericidal antibody assay has been developed as a surrogate assay, as described in the aforementioned US Patent No. 5,808,024. An N-terminally truncated V56 r200 kDa protein has been expressed in *E. coli* and antibody 20 raised to V56 r200 kDa has been shown to be bactericidal against homologous and heterologous strains of *M. catarrhalis*, thus indicating its usefulness as a vaccine antigen, as described in copending United States Patent Application No. \_\_\_\_ 25 filed \_\_\_\_ (1038-921), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. In this application, r200 kDa refers to the V56 r200 kDa protein from *M. catarrhalis* strain 4223, although other recombinant full-length and N- 30 terminally truncated 200 kDa proteins from other *M. catarrhalis* strains may be employed. Corresponding naturally-occurring proteins also may be employed.

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When under environmental stress, such as high temperature, organisms overproduce stress response or heat shock proteins (hsps). Bacterial hsps have been shown to be important immunogens, stimulating both B cells and T cells (Ref. 21). The bacterial HtrA or DegP heat shock proteins are expressed under conditions of stress and the *H. influenzae* HtrA or Hin47 protein has been shown to be a partially protective antigen in the intrabulla challenge model of otitis media (ref. 22). The HtrA proteins are serine proteases and their proteolytic activity makes them unstable. In addition, as components of a multi-component vaccine, the wild-type HtrA protein will degrade admixed antigens. The site-directed mutagenesis of the *H. influenzae* htrA gene (termed hin47) and the properties of the mutants have been fully described in US Patent No. 5,506,139 (Loosmore et al), assigned to the assignee hereof and the disclosure of which is incorporated herein by reference.

US Patent No. 5,506,139 (Loosmore et al) describes the preparation of analogs of *Haemophilus influenzae* Hin47 protein which have a decreased protease activity which is less than about 10% of that of the natural Hin47 protein and which preferably have substantially the same immunogenic properties as natural Hin47 protein. The patent also describes the isolation, purification and characterization of nucleic acid molecules encoding the Hin47 analogs. The natural Hin47 protein is immunologically conserved among non-typeable and encapsulated isolates of *H. influenzae*. The amino acid sequence of the natural Hin47 protein and the nucleotide sequence of the encoding hin47 gene

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are described in WO 94/00149 published January 6, 1994 and incorporated herein by reference.

5 The Hin47 analogs of US Patent No. 5,506,139 are prepared by deleting or replacing by a different amino acid at least one amino acid of the natural Hin47 contributing to protease activity or by inserting at least one amino acid into the natural Hin47 protein, as specifically described therein. The at least one deleted or replaced amino acid may be selected from  
10 amino acids 195 to 201 of Hin47 and specifically may be Serine-197, which may be deleted or replaced by alanine. In addition, the at least one deleted or replaced amino acid may be His-91 and may be deleted or replaced by alanine, lysine or arginine. Furthermore  
15 the at least one deleted or replaced amino acid may be Asp-121 and may be deleted or replaced by alanine.

In copending United States Patent Application No. 08/487,167 filed June 7, 1995, assigned to the assignee hereof and the disclosure of which is incorporated  
20 herein by reference, there are described multiple mutations effected at different amino acids of the natural Hin47 protein to provide the non-proteolytic Hin47 analog.

In the present invention, the mutation of  
25 histidine 91 to alanine (sometimes termed herein "H91A") is employed as illustration of the mutant Hin47 protein, although other Hin47 mutants with reduced protease activity as described in the aforementioned patent and application may be used.

30 The non-proteolytic HtrA analogue, H91A Hin47, has been shown to be a protective antigen against bacteremia caused by *H. influenzae* type b and against otitis media caused by non-typeable *H. influenzae* (ref.

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22). HtrA was found in all *H. influenzae* strains examined, including encapsulated strains. There was also evidence of cross-reactivity with a specific protein from *M. catarrhalis* on immunoblot, suggesting  
5 the possibility of an HtrA analogue in this organism.

The main goal of a prophylactic vaccine against otitis media is to prevent the establishment of nasopharyngeal colonization by including adhesins as immunogens. The *H. influenzae* HMW and Hia proteins are  
10 adhesins that have been shown to prevent colonization. However, since there may be a small percentage of *H. influenzae* strains that do not contain the *hmw* or *hia* genes, the H91A Hin47 antigen has been added to provide protection against such strains, although any other  
15 non-proteolytic analog of Hin47 may be employed. The addition of one or more *M. catarrhalis* 200 kDa adhesins provides protection against colonization by this organism. The present invention provides for a multi-component vaccine to protect against colonization and  
20 disease caused by encapsulated or unencapsulated *H. influenzae* and *M. catarrhalis* organisms.

It would be desirable to provide efficacious combination vaccines comprising *H. influenzae* and *M. catarrhalis* components containing selected relative  
25 amounts of selected antigens.

#### SUMMARY OF THE INVENTION

The present invention is directed towards the provision of a multi-component vaccine, comprising at least three antigens from *H. influenzae* and at least  
30 one antigen from *M. catarrhalis*, to protect against disease caused by *H. influenzae* and *M. catarrhalis*, including otitis media.

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In accordance with one aspect of the present invention, there is provided a multi-valent immunogenic composition for conferring protection in a host against disease caused by infection with *Haemophilus influenzae* and *Moraxella catarrhalis*, which comprises at least four different antigens, comprising at least one antigen from *Haemophilus influenzae* and at least one antigen from *Moraxella catarrhalis*, at least three of which antigens are adhesins and at least one of which adhesins is from *Moraxella catarrhalis*.

One of the antigens which is an adhesin may be a high molecular weight protein (HMW) of a non-typeable strain of *Haemophilus*, particularly an HMW1 or HMW2 protein of the non-typeable strain, which may be produced recombinantly.

Another of the antigens which is an adhesin may be a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae* or a *Haemophilus influenzae* surface fibril (hsf) protein of a typeable strain of *Haemophilus influenzae*, which may be produced recombinantly.

An antigen of *Haemophilus influenzae* which is not an adhesin may be a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae*, which may be an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of that of natural Hin47 protein.

One of the antigens which is an adhesin may be an outer membrane protein of *Moraxella catarrhalis* having an apparent molecular mass of about 200 kDa, as determined by SDS-PAGE, and may be produced recombinantly.

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In accordance with a preferred embodiment of the present invention, there is provided a multi-valent immunogenic composition for conferring protection in a host against disease caused by both *Haemophilus influenzae* and *Moraxella catarrhalis*, which comprises: (a) an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein, (b) a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae*, (c) a high molecular weight (HMW) protein of a strain of non-typeable *Haemophilus influenzae*, and (d) an outer membrane protein of *Moraxella catarrhalis* having an apparent molecular mass of about 200 kDa, as determined by SDS-PAGE.

In such composition, the Hin47, Hia, HMW and 200 kDa proteins may be present in amounts which do not impair the individual immunogenicities of the proteins, so that there is no interference between the components with respect to their individual immunogenicities.

The analog of Hin47 protein may be one in which at least one amino acid of the natural Hin47 protein contributing to protease activity has been deleted or replaced by a different amino acid and which has substantially the same immunogenic properties as natural Hin47 protein.

Such at least one amino acid may be selected from the group consisting of amino acids 91, 121 and 195 to 207 of natural Hin47 protein. Specific mutants which may be used including serine-197 replaced by alanine, Histidine-91 replaced by alanine, lysine or arginine and Asp-121 replaced by alanine.

The HMW protein of the non-typeable strain of *Haemophilus influenzae* may be a HMW1 or HMW2 protein and may be recombinantly produced. The HMW1 and HMW2 proteins are derived from the respective strains of non-typeable *Haemophilus influenzae* and possess respective molecular weights as set forth in the following Table I:

TABLE I

Molecular Weight(kDa)	Non-typeable <i>H. influenzae</i> Strain					
	12	JoyC	K21	LCDC2	PMH1	15
Mature Protein: HMW1	125	125.9	104.4	114.0	102.4	103.5
HMW2	120	100.9		111.7	103.9	121.9

The Hia and 200 kDa proteins may be produced recombinantly and may comprise N-terminal truncations, V38 rHia and V56 r200 kDa respectively.

The immunogenic composition of the invention may be further formulated with an adjuvant. Such adjuvant for use in the present invention may include (but not limited to) aluminum phosphate, aluminum hydroxide, QS21, Quil A, derivatives and components thereof, ISCOM matrix, calcium phosphate, calcium hydroxide, zinc hydroxide, a glycolipid analog, an octadecyl ester of an amino acid, a muramyl dipeptide, polyphosphazene, ISCOPREP, DC-chol, DDBA and a lipoprotein and other adjuvants. Advantageous combinations of adjuvants are described in copending United States Patent Applications No. 08/261,194 filed June 16, 1994 and 08/483,856 filed June 7, 1995, assigned to the assignee hereof and the disclosures of which are incorporated herein by reference (WO 95/34308, published November 21, 1995). The adjuvant preferably may comprise aluminum phosphate or aluminum hydroxide (collectively known as alum).

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The components of the composition may be present in appropriate quantities to provide the desired immune response. The components may be formulated as a vaccine for in vivo administration to the host. The vaccine composition may comprises:

(a) about 25 to about 100 µg of the Hin47 protein analog,

(b) about 25 to about 100 µg of the Hia protein,

(c) about 25 to about 100 µg of the HMW protein,

10 and

(d) about 25 to about 100 µg of the 200 kDa protein.

The immunogenic compositions may be formulated with other antigenic components to provide a multi-valent vaccine in which the additional antigenic component(s) confer protection against disease caused by another pathogen(s). Such additional antigens should be such that and be present in quantities that the immunogenicity of the individual components of the resulting vaccine is not impaired by other individual components of the composition. Such additional antigens preferably are purified antigens in defined quantities to produce a component vaccine.

Such additional antigens may be those traditionally found in multi-valent protective vaccines, such as diphtheria toxoid, tetanus toxoid and pertussis antigens, including pertussis toxoid, filamentous hemagglutinin, pertactin and/or agglutinogens.

30 The resulting multi-valent vaccine also may contain non-virulent poliovirus, such as inactivated poliovirus, which may be type 1, type 2 and/or type 3

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poliovirus. The multi-component vaccine further may comprise a conjugate of a tetanus or diphtheria toxoid and a capsular polysaccharide of *Haemophilus influenzae*, preferably PRP-T.

5 The invention extends to a method of immunizing a host against disease caused by infection with both *Haemophilus influenzae* and *Moraxella catarrhalis*, including otitis media, which comprises administering to the host an immunoeffective amount of the  
10 immunogenic composition provided herein.

Advantages of the present include a multi-valent vaccine that can confer protection against encapsulated and unencapsulated *Haemophilus influenzae* and *Moraxella catarrhalis* diseases in a safe and efficacious manner.

15 BRIEF DESCRIPTION OF DRAWINGS

The present invention will be further understood from the following description with reference to the drawings, in which:

Figure 1, having Panels A and B, shows the anti-H91A Hin47 immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in mice. In panel A, the H91A Hin47 + rHMW + rHia components were at a concentration of 0.3 µg each and, in panel B, they were at a concentration of 3.0 µg each. Increasing amounts  
20 of r200 kDa were added at 0, 0.3, 1.0, 3.0 and 10.0 µg;  
25

Figure 2, having Panels A and B, shows the anti-rHMW immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in mice. In panel A, the H91A Hin47 + rHMW + rHia components were at a  
30 concentration of 0.3 µg each and, in panel B, they were at a concentration of 3.0 µg each. Increasing amounts of r200 kDa were added at 0, 0.3, 1.0, 3.0 and 10.0 µg;

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Figure 3, having Panels A and B, shows the anti-rHia immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in mice. In panel A, the H91A Hin47 + rHMW + rHia components were at a concentration of 0.3 µg each and, in panel B, they were at a concentration of 3.0 µg each. Increasing amounts of r200 kDa were added at 0, 0.3, 1.0, 3.0 and 10.0 µg;

Figure 4, having Panels A, B, C and D, shows the anti-r200 kDa immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in mice. In panel A, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 0.3 or 3.0 µg each were added to 0.3 µg of r200 kDa. In panel B, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 0.3 or 3.0 µg each were added to 1.0 µg of r200 kDa. In panel C, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 0.3 or 3.0 µg each were added to 3.0 µg of r200 kDa. In panel D, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 0.3 or 3.0 µg each were added to 10.0 µg of r200 kDa;

Figure 5, having Panels A and B, shows the anti-H91A Hin47 immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in guinea pigs. In panel A, the H91A Hin47 + rHMW + rHia components were at a concentration of 25 µg each and, in panel B, they were at a concentration of 50 µg each. Increasing amounts of r200 kDa were added at 0, 25, 50 and 100 µg;

Figure 6, having Panels A and B, shows the anti-HMW immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in guinea pigs. In panel A, the H91A Hin47 + rHMW + rHia components were at a concentration of 25 µg each and, in panel B, they were

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at a concentration of 50 µg each. Increasing amount of r200 kDa were added at 0, 25, 50 and 100 µg;

Figure 7, having Panels A and B, shows the anti-Hia immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in guinea pigs. In panel A, the H91A Hin47 + rHMW + rHia components were at a concentration of 25 µg each and in panel B, they were at a concentration of 50 µg each. Increasing amounts of r200 kDa were added at 0, 25, 50 and 100 µg;

Figure 8, having panels A, B and C, shows the anti-200 kDa immune responses for H91A Hin47 + rHMW + rHia + r200 kDa combination vaccines in guinea pigs. In panel A, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 25 or 50 µg each were added to 25 µg of r200 kDa. In panel B, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 25 or 50 µg each were added to 50 µg of r200 kDa. In panel C, increasing amounts of H91A Hin47 + rHMW + rHia at 0, 25 or 50 µg each were added to 100 µg of r200 kDa;

Figure 9 shows the protection of the H91A Hin47 + rHMW + rHia + r200 kDa combination vaccine in the chinchilla model of nasopharyngeal colonization. The protection afforded by the four component vaccine is compared to that for a mono-component rHMW vaccine, a two component rHMW + H91A Hin47 vaccine, a three component rHMW + H91A Hin47 + rHia vaccine and convalescent controls;

Figure 10 illustrates the protection afforded by the H91A Hin47 + rHMW + rHia + r200 kDa combination vaccine in the chinchilla model of intrabulla challenge. The protection afforded by the four component vaccine is compared to that for a mono-

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component H91A Hin47 (H91A) vaccine, a two component rHMW + H91A Hin47 vaccine, a three component rHMW + H91A Hin47 + rHia vaccine and alum controls;

Figure 11 is a schematic illustration of the construction scheme for producing plasmid DS-2150-1 containing the gene encoding the H91 Hin47 analog;

Figure 12 is a schematic illustration of the construction scheme for producing plasmid BK-76-1-1 containing the hmw1ABC gene cluster from NTHi strain 12;

Figure 13 is a schematic illustration of a construction scheme for producing plasmid BK-96-2-11 containing the gene encoding N-truncated V38 truncated Hia from NTHi strain 11; and

Figure 14A and 14B are a schematic illustration of a construction scheme for producing plasmid pKS348 containing the gene encoding N-truncated V56 r200 kDa from *M. catarrhalis* strain 4223.

#### GENERAL DESCRIPTION OF THE INVENTION

The production and purification of recombinant *H. influenzae* antigens rHMW, rHia and H91A Hin47 have been fully described in the aforementioned US Patent Applications Nos. 09/167,568 and 09/268,347 and the aforementioned US Patent No. 5,506,139 respectively.

The production and purification of recombinant *M. catarrhalis* r200 kDa antigen has been fully described in the aforementioned US Patent Application No. \_\_\_\_ (1038-921).

Colonization of the nasopharynx is the first step in disease development for many bacterial or viral pathogens and vaccines containing adhesin molecules should protect against this first step in disease progression. The high molecular weight (HMW) proteins,

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found in approximately 75% of non-typeable *H. influenzae*, have been shown to be adhesins that are protective against colonization when administered in a vaccine composition. The HMW proteins are not present  
5 in encapsulated *H. influenzae* strains or in about 25% of non-typeable *H. influenzae* strains, thus they are not sufficient for a fully-effective vaccine having strain-wide protectivity.

The Hia/Hsf proteins also have been shown to be  
10 adhesins and are present in all encapsulated *H. influenzae* strains and in the majority of those non-typeable *H. influenzae* strains that do not produce HMW proteins. The rHia protein is protective against colonization by NTHi and against bacteremia caused by  
15 *H. influenzae* type a and type b organisms. There is a small percentage of NTHi strains that produce neither HMW nor Hia proteins.

The HtrA protein or Hin47 is found in all encapsulated and non-typeable *H. influenzae* strains.  
20 Hin47 or its non-proteolytic H91A Hin47 mutant, is protective against bacteremia caused by *H. influenzae* type b and otitis media caused by non-typeable *H. influenzae*, but it does not prevent colonization. Hin47 is proteolytic and cannot itself be used in protein  
25 formulations. A combination vaccine comprising rHMW, rHia and H91A Hin47 antigens may be formulated to protect against *H. influenzae* disease, including otitis media.

The *M. catarrhalis* 200 kDa protein is genetically  
30 and antigenically related to the Hia/Hsf family of adhesins. Immunization with r200 kDa protein elicits cross-reactive bactericidal antibodies. A combination vaccine comprising the three *H. influenzae* antigens and

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the *M. catarrhalis* r200 kDa protein may be formulated to protect against *H. influenzae* and *M. catarrhalis* disease, including otitis media.

5 The composition of multi-component vaccines is important. The vaccine components must be compatible and they must be combined in appropriate ratios to avoid antigenic interference and optimize any possible synergies. If administered with other established vaccines, they must not interfere with the protection  
10 afforded by the vaccine against other disease(s).

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The preparation, immunogenic and protective properties of a three component rHMW + rHia + H91A Hin47 vaccine have been described in US Patent Application No. 09/261,182 filed March 3, 1999,  
15 assigned to the assignee hereof and the disclosure of which is incorporated herein by reference. As described therein, rHia is combined with a two component rHMW + H91A Hin47 vaccine, the preparation, immunogenic and protective properties of which are described in  
20 copending United States Application No. 09/210,995 filed December 15, 1998, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference.

Various antigen ratios were compared for the four  
25 component H91A Hin47 + rHMW + rHia + r200 kDa vaccine and the immunogenicity was compared in two animal species. In mice, the addition of the r200 kDa component to the low dose three component vaccine (0.3 µg each), enhanced the primary anti-H91A Hin47  
30 response, however the response for the higher dose vaccine (3.0 µg each), was not affected. In guinea pigs, the anti-H91A Hin47 response for the 3 or 4

component vaccine was equivalent. In mice the anti-rHMW response to the low dose three component vaccine with or without added r200 kDa antigen was very poor. There did not appear to be either a synergistic or  
5 inhibiting effect on the anti-rHMW response upon addition of the r200 kDa component to the high dose vaccine. There was no apparent effect on the anti-rHia response upon addition of the r200 kDa component to the low or high dose three component vaccine. The immune  
10 response to the r200 kDa component with or without the added three component vaccine, was generally poorer than observed for the other components. At the 1.0 µg dose of r200 kDa, the addition of the three component vaccine at 3 µg each, decreased the immune response to  
15 r200 kDa. However, at higher doses, the anti-r200 kDa response was unaffected by the presence of the other components. In guinea pigs, the immune response to each antigen were unaffected by the presence of the other three.

20 The protection afforded by the four component vaccine was assessed in the chinchilla model of nasopharyngeal colonization. The animals were well protected against colonization, at levels equivalent to a mono-component rHMW vaccine, a two component rHMW +  
25 H91A Hin47 vaccine, or a three component rHMW + rHia + H91A Hin47 vaccine. The protection afforded by the four component vaccine was also assessed in the chinchilla intrabulla challenge model of otitis media. The animals were partially protected against otitis media, at  
30 levels equivalent to a mono-component H91A Hin47 vaccine, a two-component H91A Hin47 + rHMW vaccine, or a three-component H91A Hin47 + rHMW + rHia vaccine.

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These data demonstrate that the addition of more antigens to the vaccine has not had a deleterious effect on the protection afforded by a single component.

5 Referring to Fig. 1, there is illustrated the immune response in mice, to the H91A Hin47 antigen of a three or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 0.3 or 3.0  $\mu$ g each and r200 kDa is added at concentrations of 0, 0.3,  
10 1.0, 3.0 and 10.0  $\mu$ g. High titer antibodies are obtained in the final bleed sera for all combinations.

Referring to Fig. 2, there is illustrated the immune response in mice, to the rHMW antigen of a three or four component vaccine in which the H91A Hin47 +  
15 rHMW + rHia components are fixed at 0.3 or 3.0  $\mu$ g each and r200 kDa is added at concentrations of 0, 0.3, 1.0 3.0 and 10.0  $\mu$ g. The immune response to the low dose vaccine is very poor, but high antibody titers are obtained in the final bleed sera for the high dose  
20 three or four component vaccines.

Referring to Fig. 3, there is illustrated the immune response in mice, to the rHia antigen of a three or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 0.3 or 3.0  $\mu$ g each  
25 and r200 kDa is added at concentrations of 0, 0.3, 1.0, 3.0 and 10.0  $\mu$ g. High titer antibodies are obtained in the final bleed sera for all combinations.

Referring to Fig. 4, there is illustrated the immune response in mice, to the r200 kDa antigen of a one or  
30 four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 0, 0.3 or 3.0  $\mu$ g each and r200 kDa is added at concentrations of 0.3, 1.0, 3.0

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and 10.0  $\mu\text{g}$ . The immune response to the r200 kDa component is very poor at the 0.3  $\mu\text{g}$  and 1.0  $\mu\text{g}$  doses. At the higher doses of 3 or 10  $\mu\text{g}$  of r200 kDa, high antibody titers are obtained for the final bleed sera, irrespective of the presence or absence of the other components.

Referring to Fig. 5, there is illustrated the immune response in guinea pigs, to the H91A Hin47 antigen of a three or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 25 or 50  $\mu\text{g}$  each and r200 kDa is added at concentrations of 0, 25, 50 or 100  $\mu\text{g}$ . High titer antibodies are obtained in the final bleed sera from all combinations.

Referring to Fig. 6, there is illustrated the immune response in guinea pigs, to the rHMW antigen of a three or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 25 or 50  $\mu\text{g}$  each and r200 kDa is added at concentrations of 0, 25, 50 or 100  $\mu\text{g}$ . High titer antibodies are obtained in the final bleed sera from all combinations.

Referring to Fig. 7, there is illustrated the immune response in guinea pigs, to the rHia antigen of a three or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 25 or 50  $\mu\text{g}$  each and r200 kDa is added at concentrations of 0, 25, 50 or 100  $\mu\text{g}$ . High titer antibodies are obtained in the final bleed sera from all combinations.

Referring to Fig. 8, there is illustrated the immune response in guinea pigs, to the r200 kDa antigen of a one or four component vaccine in which the H91A Hin47 + rHMW + rHia components are fixed at 0, 25 or 50

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µg each and r200 kDa is added at concentrations of 25, 50 or 100 µg. High titer antibodies are obtained in the final bleed sera from all vaccines.

Referring to Fig. 9, there is illustrated the protection afforded by the four component H91A Hin47 + rHMW + rHia + r200 kDa vaccine against nasopharyngeal colonization in a chinchilla model. The protection is comparable to that afforded by a mono-valent rHMW vaccine, a two component rHMW + H91A Hin47 vaccine and a three component rHMW + rHia + H91A Hin47 vaccine.

Referring to Fig. 10, there is illustrated the protection afforded by the four component H91A Hin47 + rHMW + rHia + r200 kDa vaccine against middle ear infection in a chinchilla model. The protection is comparable to that afforded by a mono-valent rHMW vaccine, a two component H91A Hin47 + rHMW vaccine, and a three component H91A Hin47 + rHMW + rHia vaccine.

#### EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry, immunology and fermentation technology used, but not explicitly described in this disclosure

and these Examples, are amply reported in the scientific literature and are well within the ability of those skilled in the art.

#### Example 1

5 This Example describes the preparation of the H91A Hin47 vaccine component.

The H91A Hin47 mutant was prepared as described in the aforementioned US Patent No. 5,506,139. Briefly, an oligonucleotide 5' ATCAATAACAGCATTATTGGT 3' (SEQ ID NO:  
10 1) was synthesized which would change the Histidine residue at 91 to an Alanine (ref. 17).

Plasmid JB-1276-1-2 is a pUC-based plasmid containing the T7/hin47 gene on an EcoR I fragment and was used to clone the hin47 gene into M13mp18 for site-  
15 directed mutagenesis with the In Vitro Site-Directed Mutagenesis kit from Amersham. The preparation of plasmid JB-1276-1-2 is described in USP 5,506,139. The mutation of the His91 codon to Ala91 was confirmed by local sequencing. The H91A mutant hin47 gene was  
20 subcloned into pT7-7 to generate plasmid DS-1277-19 (Fig. 11).

The H91A Hin47 expression plasmid (DS-1277-19) utilizes ampicillin selection. The T7/H91A hin47 gene was cloned into pBR328 so that tetracycline selection  
25 could be used. Vector DS-1312-12 was thus a pBR328-based plasmid which contained the T7/H91A hin47 gene sequences between EcoR I and Cla I sites, having functional ampicillin and tetracycline resistance genes and containing a repeat of the Hind III - BamH I  
30 sequences which are found in both pBR328 and pEVvrf1.

A new plasmid based upon DS-1312-12 was constructed which utilizes kanamycin selection. The construction scheme is shown in Figure 11. Plasmid DNA

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from DS-1312-12 was digested with *Hind* III generating two fragments. The larger 5.9 kb fragment contained a promoterless *tetR* gene, the *ampR* gene and the T7/H91A *hin47* gene and was re-ligated on itself creating vector DS-2140-3. Plasmid DS-2140-3 was digested with *Pst* I and the *kanR* gene from plasmid pUC-4K (P-L Biochemicals) was inserted into the *Pst* I site, generating plasmid DS-2150-1 which is *kanR* and sensitive to both ampicillin and tetracycline.

Plasmid DNA from DS-2150-1 was prepared from a 50 mL culture using a protocol based upon the Holmes and Quigley procedure (ref. 23) and including extractions with phenol and chloroform. *E. coli* BL21(DE3) cells were made electrocompetent as follows. Briefly, 10 mL of overnight culture were inoculated into 500 mL of YT medium and the cells were grown at 37°C with shaking until they reached an  $A_{620}=0.540$ . The culture was chilled on ice for 30 min., spun at 5K rpm for 15 min., and the cell pellet resuspended in 500 mL ice cold sterile water. The cell suspension was centrifuged as before and the cell pellet resuspended in 250 mL ice cold sterile water. The cell suspension was spun again, and the cells were resuspended in 10 mL of 10% glycerol. The glycerol suspension was spun, and the cells were resuspended in 1.5 mL of 10% glycerol, aliquotted as 40  $\mu$ l samples, and stored at -70°C.

One aliquot of electrocompetent BL21(DE3) cells was thawed on ice and approximately 9 ng of DS-2150-1 DNA was added. Samples were incubated on ice for 3 min. then transferred to a -20°C BioRad Gene Pulser electrode cuvette and subjected to an electric pulse. 900  $\mu$ l of SOC medium were added and the mixture transferred to a culture tube where it was incubated at

37°C for 1 hour before being plated onto YT agar containing 25 µg/mL kanamycin. The plate was incubated overnight at 37°C and single colonies were used for expression studies.

5 Individual clones were grown in NZCYM medium to an  $A_{600\text{ nm}}$  of approximately 0.3 and lactose was added to 1% to induce expression. Cells were grown for 4 hours, then harvested and analysed by SDS PAGE. Clone DS-2171-1-1 was chosen as a representative clone which  
10 expressed high levels of H91A Hin47.

The *E. coli* containing DS-2171-1-1 was grown in 2 X 2 L flasks containing 250 mL of ECGM (containing 8 g/L glucose, pH 6.5) and incubated by shaking at 37°C for approximately 9 hours in the dark at 250 rpm. The  
15 culture fluid (2 x 250 mL) was inoculated into a 10 L fermentor and the culture grown at 37°C. After approximately 10 hours of incubation, 1% lactose (final concentration) is added for induction, followed by an additional 4 hours incubation.

20 The culture fluid was harvested into sterile transfer bottles and concentrated and diafiltered by cross-flow filtration against 50 mM Tris/HCl buffer, pH 8.0. The cells in the concentrate are lysed using a high-pressure homogenizer (2 passes at 15,000 psi) to  
25 release the H91A Hin47 protein. The cell debris was removed by centrifugation at 15,000 rpm for 1.5 hours. The supernatant was further clarified by centrifugation and filtered through a 0.22 µm dead-end filter. Products may be stored frozen at -70°C until further  
30 processing.

Sodium chloride (NaCl) was added to the clarified sample to a final concentration of 100 mM. The sample was then purified on an anion exchange chromatography.

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column (TMAE-Fractogel) equilibrated with 50 mM Tris pH 8.0 containing 100 mM NaCl. The H91A Hin47 protein was obtained in the run-through.

The aqueous layer was loaded onto a ceramic  
5 hydroxyapatite type 1 (CHTP-1) column equilibrated with 10 mM sodium phosphate buffer pH 8.0. The column was then washed with 150 mM sodium phosphate buffer pH 8.0 and H91A Hin47 was eluted with 175 mM sodium phosphate buffer, pH 8.0 containing 1 M NaCl.

10 The H91A Hin47 purified protein was concentrated using a 10 kDa molecular weight cut-off membrane followed by diafiltration with approximately 10 volumes of phosphate buffered saline (PBS), pH 7.5.

The H91A Hin47 purified protein in PBS was passed  
15 through a Q600 Sartobind membrane adsorber. After passing the solution, the membrane was regenerated using 1.0 M KCl/1.0 M NaOH followed by washing with 1 M KCl then equilibrating with PBS. The process was repeated twice. The concentrated diafiltered H91A Hin47  
20 protein was sterile filtered through a 0.22  $\mu$ m membrane filter. Sterile H91A Hin47 protein was adjuvanted with aluminum phosphate. The adsorbed purified concentrate was diluted to produce the adsorbed bulk at 400  $\mu$ g/mL.

#### Example 2

25 This Example describes the preparation of the rHMW vaccine component.

The production and purification of the rHMW protein has been described in the aforementioned copending United States Patent Application No. 09/167,568 filed  
30 October 7, 1998.

Briefly, plasmid pHMW1-15 (ref. 13) contains a Xba I site within the T7 promoter sequence and a unique BamH I site within the coding sequence of the mature

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HMW1A protein of non-typeable *Haemophilus* strain 12. The 1.8 kb *Xba* I-*Bam*H I fragment of pHMW1-15 was deleted and replaced by an approximately 114 bp *Xba* I-*Bam*H I fragment generated from oligonucleotides. The resultant  
 5 11.3 kb plasmid, DS-1046-1-1, thus contains the T7 promoter joined in frame with the *hmw1ABC* operon that encodes the mature 125 kDa HMW1A protein (Fig. 12).

Plasmid DS-1046-1-1 contains the T7 *hmw1ABC* gene cassette and has a unique *Bgl* II site outside the  
 10 coding region of the mature HMW1A gene. Plasmid DS-2224-1-4 contains the *E. coli* *cer* gene located on a *Bam*H I fragment. This fragment was isolated and ligated into the *Bgl* II site of plasmid DS-1046-1-1 to produce plasmid BK-35-4 (Fig. 12). The kanamycin resistance  
 15 cassette was excised from pUC 4K by *Sal* I restriction and ligated into the *Sal* I restricted BK-35-4 plasmid to produce plasmid BK-76-1-1 (Fig. 12).

Plasmid DNA from BK-76-1-1 was prepared from a 50 mL culture using a protocol based upon the Holmes and  
 20 Quigley procedure (ref. 23) and including extractions with phenol and chloroform. Plasmid DNA was introduced into *E. coli* BL21(DE3) cells by electroporation using a BioRad apparatus. Strains were grown at 37°C in NZCYM medium to an optical density of  $A_{578}=0.3$ , then induced by  
 25 the addition of lactose to 1.0% for 4 hours. Samples were adjusted to 0.2 OD/ $\mu$ l with SDS-PAGE lysis + loading buffer and the same amount of protein sample was loaded onto SDS-PAGE gels. Clone BK-116-1-1 was chosen as a representative clone that expressed good levels of rHMW.

30 Recombinant HMW protein was expressed as inclusion bodies in *E. coli*, and was purified as follows. *E. coli* cell pellets from 500 ml culture were resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M

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NaCl, and disrupted by sonication. The extract was centrifuged at 20,000 g for 30 min and the resultant supernatant was discarded. The pellet was further extracted, in 50 ml of 50 mM Tris-HCl, pH 8.0  
5 containing 0.5% Triton X-100 and 10 mM EDTA, then centrifuged at 20,000 g for 30 min, and the supernatant was discarded. The pellet was further extracted in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 1 % octylglucoside, then centrifuged at 20,000 g for 30  
10 min, and the supernatant was discarded.

The resultant pellet, obtained after the above extractions, contains the inclusion bodies. The pellet was solubilized in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. Twelve ml of 50  
15 mM Tris-HCl, pH 8.0 was added to this solution and the mixture was centrifuged at 20,000 g for 30 min. The supernatant was precipitated with polyethylene glycol (PEG) 4000 at a final concentration of 7%. The resultant pellet was removed by centrifugation at  
20 20,000 g for 30 min and the supernatant was precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 50% saturation. After the addition of  $(\text{NH}_4)_2\text{SO}_4$ , the solution underwent phase separation with protein going to the upper phase, which was then subjected to centrifugation at 20,000 g for 30  
25 min. The resultant pellet was dissolved in 2 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine HCl and 5 mM DTT and the clear solution was purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine HCl. The  
30 fractions were analysed by SDS-PAGE and those containing the purified rHMW1 were pooled and dialysed overnight at 4°C against PBS, then centrifuged at 20,000 g for 30 min. The protein remained soluble

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under these conditions and glycerol was added to the rHMW1 preparation at a final concentration of 20% for storage at -20°C.

The concentration of a rHMW vaccine component was adjusted to 400  $\mu\text{g ml}^{-1}$  in PBS (pH 7.3) and was adjuvanted with aluminum phosphate to a final concentration of 3  $\mu\text{g ml}^{-1}$ . Different doses were prepared by diluting the stock with 3  $\mu\text{g ml}^{-1}$  aluminum phosphate in water.

#### 10 Example 3

This Example illustrates the preparation of the rHia vaccine component.

The production and purification of the rHia protein has been described in the aforementioned copending United States Patent Application No. 09/268,347.

Briefly, plasmid DS-1843-2 is a pBR328-based plasmid in which a multiple cloning site and two transcription terminators have been introduced on oligonucleotides, between the *EcoR* I and *Pst* I sites, thus destroying both the chloramphenicol and ampicillin resistance genes (Fig. 6B). The kanamycin resistance gene from pUC-4K was inserted at the *Sal* I site, to generate plasmids DS-2147-1 that is kanamycin resistant and tetracycline sensitive. Plasmid DS-2224-1-4 is a pUC plasmid containing a synthetic *E. coli* *cer* gene (ref. 15) constructed from oligonucleotides and flanked by *BamH* I sites. The 290 bp *BamH* I fragment of the *cer* gene was inserted into the *BamH* I site of DS-2147-1 creating plasmid BK-2-1-2. This pBR-based plasmid thus contains a multiple cloning site, the kanamycin resistance gene and the *cer* gene. Plasmid BK-2-1-2 was

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linearized with *Bgl* II and dephosphorylated. Plasmid DS-2186-2-1 was digested with *Bgl* II and *Bam*H I and the 3.6 kb T7 V38 *hla* fragment was inserted into BK-2-1-2, creating plasmid BK-96-2-11 (Fig. 13).

5 Plasmid DNA from BK-96-2-11 was prepared from a 50 ml culture using a protocol based upon the Holmes and Quigley procedure (ref. 23) and including extractions with phenol and chloroform. Plasmid DNA was introduced into electrocompetent *E. coli* BL21 (DE3) cells by  
10 electroporator using a BioRad electroporator. Strains were grown at 37°C in NZCYM medium using the appropriate antibiotic selection to an optical density of  $A_{578}$  of 0.3 before induction by the addition of lactose to 1.0% for 4 hours. Samples were adjusted to  
15 0.2 OD/ $\mu$ l with SDS-PAGE lysis + loading buffer and the same amount of each protein sample was loaded onto SDS-PAGE gels. Clone BK-131-1-1 was chosen as a representative clone that expressed good levels of V38 rHia (Fig. 13).

20 Recombinant truncated Hia protein was expressed as inclusion bodies in *E. coli* and was purified as follows. *E. coli* cell pellets from 500 ml culture were resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and disrupted by sonication.  
25 The extract was centrifuged at 20,000 g for 30 min and the resultant supernatant was discarded. The pellet was further extracted, in 50 ml of 50 mM Tris-HCl, pH 8.0 containing 0.5% Triton X-100 and 10 mM EDTA, then centrifuged at 20,000 g for 30 min, and the supernatant  
30 was discarded. The pellet was further extracted in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 1% octylglucoside, then centrifuged at 20,000 g for 30 min, and the supernatant was discarded.

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The resultant pellet obtained after the above extractions contains the inclusion bodies. The pellet was solubilized in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. Twelve ml of 50 mM Tris-HCl, pH 8.0 was added to this solution and the mixture was centrifuged at 20,000 g for 30 min. The supernatant was precipitated with polyethylene glycol (PEG) 4000 at a final concentration of 7%. The resultant pellet was removed by centrifugation at 20,000 g for 30 min and the supernatant was precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 50% saturation. The  $(\text{NH}_4)_2\text{SO}_4$  precipitate was collected by centrifugation at 20,000 g for 30 min. The resultant pellet was dissolved in 2 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine HCl and 5 mM DTT and the clear solution was purified on a Superdex 200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 2 M guanidine HCl. The fractions were analysed by SDS-PAGE and those containing the purified rHia were pooled and dialysed overnight at 4°C against PBS, then centrifuged at 20,000 g for 30 min. The protein remained soluble under these conditions and glycerol was added to the rHia preparation at a final concentration of 20% for storage at -20°C.

The concentration of the rHia vaccine component was adjusted to 400  $\mu\text{g ml}^{-1}$  in PBS (pH 7.3) and was adjuvanted with aluminum phosphate to a final concentration of 3  $\text{mg ml}^{-1}$ . Different doses were prepared by diluting the stock with 3  $\text{mg ml}^{-1}$  aluminum phosphate in  $\text{H}_2\text{O}$ .

Example 4

This Example illustrates the preparation of the r200 kDa vaccine component.

The production and purification of the r200 kDa protein has been described in the aforementioned copending United States Patent Application No. \_\_\_\_\_ filed \_\_\_\_\_ (1038-921).

USP 5,808,024, assigned to the assignee hereof and the disclosure of which is incorporated herein by reference, and WO 96/34960 describe the isolation from genomic a *M. catarrhalis* genomic library in phage lambda clone 8II which expressed the about 200 kDa protein. DNA was extracted and a series of plasmid vectors was prepared from DNA fragments. Plasmid pKS348 was constructed as shown schematically in Figures 14 and 14B. Plasmid pKS47, containing a 1-1 kb *KpnI/XhoI* fragment, was digested with *XhoI* and *KpnI* and separated by agarose gel electrophoresis. The 1.1 kb fragment was isolated from the gel and inserted into plasmid pKS5 containing a 4.9 kb *XhoI/SalI* fragment, which had previously been digested with the same two enzymes and purified to form pKS80. An about 5.8 kb *PstI* fragment from pKS80 was inserted into pT7-7 vector (ref. 24) that had been digested with *PstI* and dephosphorlated. The orientation of the insert was determined by restriction enzyme analysis and pKS122 was chosen for further construction (see Figure 14A).

An about 500 bp 5' region of the 200 kDa gene was PCR amplified from *M. catarrhalis* strain 4223 from chromosomal DNA using primers 5471.KS (CGCTCGCTGTCCATATGATCGGTGCAACGCTCA - SEQ ID No: 2) and 4257.KS (GACCCTGTGCATATGACATGGCT - SEQ ID No: 3), using the condition described in the aforementioned US Patent

Application No. \_\_\_\_\_ (1038-921). The PCR-product was digested with *NdeI*, purified and inserted into *NdeI* digested and dephosphorylated pKS122 to provide pKS348 (see Figure 14B). Plasmid pKS348 was confirmed by  
5 restriction enzyme analyses and by sequencing of the PCR-amplified DNA piece and its joint regions. Such plasmid contains nucleic acid encoding an N-truncated about 200 kDa protein in which the codon encoding V56 amino acid is replaced by a start codon encoding M56  
10 amino acid (M56 r200 kDa protein).

A single colony of *E. coli*, BL21(DE3)/pLySS, (KS358) which carried pKS348, was suspended in 5 ml of BHI broth containing Amp (100  $\mu$ M) and 0.4% of glucose, and cultured overnight at 37°C. 2.5 ml of the overnight  
15 culture was added to 250 ml of LB (luria-Bertani) broth containing Amp (100  $\mu$ M) and Cm (50  $\mu$ M), and grown with shaking at 37°C to  $A_{600} = 0.26$  to 0.44. Gene expression from the cultures was induced by addition of IPTG (final concentration: 4 mM). The bacteria were grown  
20 and harvested at different time points by centrifugation. The expression of the 200 kDa protein gene in the culture was confirmed by SDS-PAGE analysis using Coomassie Blue staining and by Western blot analysis using guinea pig anti-200 kDa protein serum,  
25 as described in USP 5,808,024 and WO 96/34960.

When *E. coli* BL21(DE3)/pLySS was transformed with pKS348, transformants grew well even on LB agar plates and in LB broth containing antibiotics at 37°C. After induction with IPTG, these clones produced a large  
30 amount of the N-terminally truncated r200 kDa protein. The N-terminally truncated r200 kDa protein was

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purified from the *E. coli* culture, as shown in Figure 13.

*E. coli* cell pellets were obtained from the cultures prepared by centrifugation and were resuspended in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl, and disrupted by sonication. The sonicate was centrifuged at 20,000 xg for 30 min. and the resultant supernatant was discarded. The pellet was extracted, in 50 ml of 50 mM Tris-HCl, pH 8.0 containing 0.5% Triton X-100 and 10 mM EDTA, then centrifuged at 20,000 xg for 30 min. and the supernatant was discarded. The pellet was further extracted in 50 ml of 50 mM Tris-HCl, pH 8.0, containing 1% octylglucoside, then centrifuged at 20,000 xg for 30 min. and the supernatant was discarded.

The resultant pellet contained the inclusion bodies. The pellet was solubilized in 6 ml of 50 mM Tris-HCl, pH 8.0, containing 6 M guanidine and 5 mM DTT. Twelve ml of 50 mM Tris-HCl, pH 8.0 was added, the mixture centrifuged at 20,000 xg for 30 min. and the pellet discarded. The supernatant was precipitated by adding polyethylene glycol (PEG) 4000 at a final concentration of 5% and incubated at 4°C for 30 min. the resultant pellet was removed by centrifugation at 20,000 xg for 30 min. The supernatant was then precipitated by  $(\text{NH}_4)_2\text{SO}_4$  at 50% saturation at 4°C overnight. After the addition of  $(\text{NH}_4)_2\text{SO}_4$ , the solution underwent phase separation with protein going to the upper phase (as judged by the cloudiness of the layer). The upper phase was collected, then subjected to centrifugation at 20,000 xg for 30 min. The resultant

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30 Briefly, vaccines were prepared that comprised combination of rH1a with a two-component H91A H1n47 and rH1MW vaccine, prepared as described in copending United

States Patent Application No. 09/210,995. The components were combined on day 0, mixed overnight at 4°C and aliquotted on day 1. The combined vaccines were stored at 4°C throughout the immunization period.

- 5 Vaccines were prepared that comprised the following combinations of r200 kDa with the three component vaccine as in Table II:

TABLE II

µg r200 kDa→ µg 3 Component↓	0	0.3	1.0	3.0	10.0	25	50	100
0		m	m	m	m	gp	gp	gp
0.3 + 0.3 + 0.3	m	m	m	m	m			
3.0 + 3.0 + 3.0	m	m	m	m	m			
25 + 25 + 25	gp					gp	gp	gp
50 + 50 + 50	gp					gp	gp	gp

Notes: 3 component refers to H91A Hin47 + rHMW + rHia  
 m indicates the vaccine was used to immunize mice.  
 gp indicates that the vaccine was used to immunize guinea pigs.

Vaccine components in Table II were combined on day 0, mixed overnight at 4°C and aliquotted on day 1.

- 10 All vaccines were stored at 4°C throughout the immunization period.

#### Example 6

- This Example describes the analysis of the immunogenicity of the multi-component vaccines in  
 15 animals.

The immunogenicity of a three-component vaccine comprising H91A Hin47 + rHMW + rHia, has been described

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in the aforementioned copending United States Patent Application No. 09/261,182.

Groups of five BALB/c mice (Charles River, Quebec) were immunized subcutaneously (s.c.) on days 1, 29 and 5 43 with one of the mouse vaccines described in Example 5. Blood samples were taken on days 0, 14, 28, 42, and 56.

Groups of five Hartley outbred guinea pigs (Charles River, Quebec) were immunized intramuscularly 10 (i.m.) on days 1, 29 and 43 with one of the guinea pig vaccines described in Example 5. Blood samples were taken on days 0, 14, 28, 42, and 56.

Anti-H91A Hin47, anti-rHMW, anti-rHia and anti-r200 kDa IgG antibody titers were determined by antigen 15 specific enzyme linked immunosorbent assays (ELISAs). Microtiter wells (NuncMAXISORB, Nunc, Denmark) were coated with 100  $\mu$ l of protein solution ( $0.2 \mu\text{g ml}^{-1}$ ). The secondary antibodies used were affinity-purified F(ab'), fragments of goat anti-mouse IgG (Fc-specific) 20 or anti-guinea pig IgG (Fc-specific) antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs, Mississauga, Ontario). The reactions were developed using tetramethylbenzidine (TMB/H<sub>2</sub>O<sub>2</sub>, ADI, Mississauga, Ontario) and absorbancies 25 were measured at 450 nm (using 540 nm as a reference wavelength) in a Flow Multiskan MCC microplate reader (ICN Biomedicals, Mississauga, Ontario). The reactive titer of an antiserum was defined as the reciprocal of the dilution consistently showing a two-fold increase 30 in absorbance over that obtained with the pre-bleed serum sample.

The results of the immunogenicity studies are illustrated in Figures 1 to 8. As shown in Figure

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1, Panels A and B, the final bleed sera obtained from mice immunized with 0.3  $\mu$ g, or 3.0  $\mu$ g each of H91A Hin47 + rHMW + rHia with 0, 0.3, 1.0, 3.0 or 10.0  $\mu$ g of added r200 kDa, all had high antibody titers to H91A  
5 Hin47 component. With the low dose three component vaccine, there may be a synergistic effect on the primary anti-H91A Hin47 response with added r200 kDa.

As shown in Figure 2, panels A and B, the final bleed sera obtained from mice immunized with 3.0  $\mu$ g  
10 each of H91A Hin47 + rHMW + rHia with 0, 0.3, 1.0, 3.0 or 10.0  $\mu$ g of added r200 kDa, all had high titer antibodies to the rHMW component. The low dose vaccine was poorly immunogenic. There was no apparent enhancing or inhibiting effect on the anti-rHMW response with the  
15 addition of the r200 kDa component.

As shown in Figure 3, panels A and B, the final bleed sera obtained from mice immunized with 0.3  $\mu$ g each of H91A Hin47 + rHMW + rHia with 0, 0.3, 1.0, 3.0 or 10.0  $\mu$ g of added r200 kDa, all had high titer  
20 antibodies to the rHia component. There was no apparent enhancing or inhibiting effect on the anti-rHia response with the addition of the r200 kDa component.

As shown in Figure 4, panels A to D, the final bleed sera obtained from mice immunized with 10.0  $\mu$ g of  
25 r200 kDa added to 0, 0.3  $\mu$ g or 3.0  $\mu$ g each of H91A Hin47 + rHMW + rHia, all had high titer antibodies to the r200 kDa component. There was no apparent enhancing or inhibiting effect on the anti-r200 kDa response with the addition of the other vaccine components. However,  
30 at lower doses of r200 kDa, the vaccine was poorly immunogenic and at the 1.0  $\mu$ g dose, there is a

statistically significant inhibitory effect of the added components on the anti-r200 kDa response.

The data in mice demonstrate the preferred nature of the composition of a multi-component vaccine by preventing suppression of responses to individual antigens.

The immunogenicity in guinea pigs is illustrated in Figures 5 to 8. As shown in Figure 5, panels A and B, the final bleed sera obtained from guinea pigs immunized with 25 µg or 50 µg each of H91A Hin47 + rHMW + rHia with 0, 25, 50 or 100 µg of added r200 kDa, all had high titer antibodies to the H91A Hin47 component. There was no apparent enhancing or inhibiting effect on the anti-H91A Hin47 response upon the addition of the r200 kDa antigen.

As shown in Figure 6, panels A and B, the final bleed sera obtained from guinea pigs immunized with 25 µg or 50 µg each of H91A Hin47 + rHMW + rHia with 0, 25, 50 or 100 µg of added r200 kDa, all had high titer antibodies to the rHMW component. There was no apparent enhancing or inhibiting effect on the anti-rHMW response upon the addition of the r200 kDa antigen.

As shown in Figure 7, panels A and B, the final bleed sera obtained from guinea pigs immunized with 25 µg or 50 µg each of H91A Hin47 + rHMW + rHia with 0, 25, 50 or 100 µg of added r200 kDa, all had high titer antibodies to the rHia component. There was no apparent enhancing or inhibiting effect on the anti-rHia response upon the addition of the r200 kDa antigen.

As shown in Figure 8, panels A, B and C, the final bleed sera obtained from guinea pigs immunized with 25, 50 or 100 µg of r200 kDa with added 0, 25 or 50 µg each

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of H91A Hin47 + rHMW + rHia, all had high titer antibodies to the r200 kDa component. There was a statistically significant effect on the anti-r200 kDa antibody titers for some of the interim bleeds, when  
5 increasing amounts of the other components were added. However, there was no significant effect on the final bleeds.

Example 7

This Example describes the protective ability of a  
10 multi-component vaccine in animal models of disease.

In young chinchillas, it has been demonstrated that nasopharyngeal colonization with non-typeable *H. influenzae* leads to otitis media (ref. 17). rHMW is partially protective in a chinchilla nasopharyngeal  
15 colonization challenge model, as described in the aforementioned US Patent Application No. 09/167,568. In this model, animals are immunized i.m. on days 0, 14 and 28 with 25, 50 or 100 µg of rHMW adsorbed to alum, and challenged on day 44 with 10<sup>8</sup> cfu of live bacteria  
20 delivered intranasally (50 µl per nares). Nasopharyngeal lavage is performed 4 days post challenge using 1 ml of sterile saline as wash. 25 µl of wash is plated onto chocolate agar in the presence of streptomycin and the plates incubated at 37°C for 24  
25 h. (The challenge stain was made streptomycin resistant by serial passaging, in order to facilitate the quantitation of recovered bacteria in the presence of natural flora that are killed by the streptomycin.) Convalescent animals or those mock-immunized with alum  
30 alone, are used as controls. For the multi-component vaccine study, 50 µg of each of H91A Hin47, rHMW, rHia and r200 kDa was mixed as described in Example 5 and

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chinchillas were immunized as described above. The results of the protection study are shown in Figure 9 which indicates that there is still excellent protection afforded in the nasopharyngeal colonization challenge model by the combination of rHMW + rHia + H91A Hin47 + r200 kDa.

H91A Hin47 is partially protective in the chinchilla model of otitis media, as described in the aforementioned US Patent No. 5,506,139. In this model, 1 to 2 year old chinchillas (Moulton Chinchilla Ranch, Rochester, Minnesota) are immunized i.m. on days 0, 14 and 28 with 30 µg of H91A Hin47 adsorbed to alum, and challenged on day 44 with 50 to 350 cfu of live organisms delivered into the middle ear space via the epitympanic bulla (ref. 14). Animals are monitored by tympanometry and middle ear fluid is collected 4 days post challenge, mixed with 200 µl of BHI medium and dilutions plated onto chocolate agar plates that are incubated for 24 h at 37°C. Convalescent animals or those mock-immunized with alum alone, are used as controls. For the multi-component vaccine study, 50 µg of each of H91A Hin47, rHMW, rHia and r200 kDa were combined as described in Example 5 and chinchillas were immunized as described above. The results of the protection study are shown in Figure 10 which indicates that there is still partial protection afforded in the intrabulla challenge model by the combination of rHMW + rHia + H91A Hin47 + r200 kDa.

#### Example 8

This Example illustrates the bactericidal properties of the composition.

There is no relevant animal model for infection by *Moraxella catarrhalis* but a bactericidal antibody assay has been developed as a surrogate assay. Briefly, *M. catarrhalis* strain 4223 was cultured overnight in brain  
5 heat infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C. The overnight culture was subcultured into 10 ml BHI broth and grown to  $A_{578} = 0.5$ .

Bacteria were diluted ( $10^{-3}$  or  $10^{-4}$ ) in Veronal buffered saline (VBS, pH 7.6) containing 140 mM NaCl,  
10 93 mM  $\text{NaHCO}_3$ , 2 mM Na-barbiturate, 4 mM bartiuric acid, 0.5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.4 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 0.1% bovine serum albumin. Guinea pig anti-r200 kD serum and pre-immune control serum were heated at 56°C for 30 min. to  
15 inactivate endogenous complement. Serum and antiserum were diluted in VBS, and placed on ice.

Twenty-five  $\mu\text{l}$  of diluted pre-immune serum or test antiserum were added to the wells of a 96-well Nunclon microtitre plate (Nunc, Roskilde, Denmark). Twenty-five  
20  $\mu\text{l}$  of diluted bacterial cells were added to each of the wells. A guinea pig complement (BioWhittaker, Walkerville, MD) was diluted 1:10 in VBS, and 25  $\mu\text{l}$  portions were added to each well. The plates were incubated for 60 min., gently shaking at 70 rpm on a rotary platform. Fifty  $\mu\text{l}$  of each reaction mixture were  
25 plated onto Mueller Hinton agar plates (Becton-Dickinson, Cockeysville, MD). The plates were incubated at 37°C for 24 hours, and then left at room temperature for a further 24 hours. The number of colonies per plate was counted, and averaged values of colonies per  
30 plate were estimated from duplicate pairs.

When pre-immune serum plates were compared with PBS control plates (no serum), pre-immune serum had no

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bactericidal effect on the homologous strain 4223. Therefore, it was assumed that the number of colonies per plate on pre-immune serum plates represented 100% viability for each strain and percent bactericidal killing was calculated as follows:

$$100\% - \frac{\text{average number of colonies per plate in anti-r200 kD antiserum group} \times 100}{\text{average number of colonies per plate in pre-immune serum group}}\%$$

Using this assay, the relative bactericidal antibody activity of anti-r200 kDa and anti-4 component (H91A Hin47 + rHMW + rHia + r200 kDa), antisera were compared and found to be equivalent. These data, set forth in Tables III and IV below, indicate that there is no adverse effect on the bactericidal activity of anti-r200 kDa antibody when antibodies to additional antigens are present.

#### SUMMARY OF THE DISCLOSURE

In summary of this disclosure, the present invention provides a multi-valent vaccine against disease caused by both *Haemophilus influenzae* and *Moraxella catarrhalis*, including otitis media, which has a wide spectrum of efficacy, and comprising three different antigens of *Haemophilus influenzae*, two of which different antigens is a adhesin, and an antigen of *Moraxella catarrhalis*, which is antigenically related to one of the antigens of *Haemophilus influenzae*. Modifications are possible within the scope of the invention.

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TABLE III

PERCENT KILLING OF 4223 BY GP 1409-13  $\alpha$  r200kD (alum)  
AND GP 1449-53 rH91,  $\alpha$  Hia, HMW, r200kD (alum)

	ANTISERUM DILUTION						
	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
GP 1409-13 $\alpha$ r200kD (alum)	83%	57%	32%	25%	20%		
GP 1449-53 $\alpha$ rHia, rHMW, r200kD, rH91A (alum)	71%	47%	15%	17%	5%		
GP 1338-43 $\alpha$ r200kD			100%	99%	99%	96%	78%

LM613, 11.12.98

Based on  $\log_{10} = -4$  dilution of 4223

Ave prebleed colony count was 482

TABLE IV

PERCENT KILLING OF 4223 BY COMBINATION ANTISERA

Antiserum dilution:	Bact. Dil = $\log_{10} -4$				Bact. Dil = $\log_{10} -3$			
	1/64	1/256	1/1024	1/4096	1/64	1/256	1/1024	1/4096
GP 1631-36 $\alpha$ rHia rH91A, rHMW, r200kD (FCA/FIA)	72%	41%	49%	43%	12%	0%	30%	9%
GP 1637-40 $\alpha$ r200kD (FCA/FIA)	90%	76%	59%	38%	88%	71%	26%	3%
GP 1651-55 $\alpha$ rHia rH91A, rHMW, r200kD (alum)	79%	37%	66%	43%	65%	0%	5%	0%
GP 1656-60 $\alpha$ r200kD (alum)	86%	79%	74%	54%	77%	25%	22%	4%
GP 1338-43 $\alpha$ r200kD (control)	97%	95%	84%	59%	92%	89%	77%	47%

LM615, 16.12.98

Ave. prebleed colony count at  $\log_{10} -4$  dilution of 4223 was 720

Ave. prebleed colony count at  $\log_{10} -3$  dilution of 4223 was 58

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CLAIMS

What we claim is:

1. A multi-valent immunogenic composition for conferring protection in a host against disease caused by both *Haemophilus influenzae* and *Moraxella catarrhalis*, which comprises:

at least four different antigens comprising at least one antigen from *Haemophilus influenzae* and at least one antigen from *Moraxella catarrhalis*, at least three of which antigens are adhesins and at least one of which adhesins is from *Moraxella catarrhalis*.

2. The immunogenic composition of claim 1 wherein one of said antigens which is an adhesin is a high molecular weight (HMW) protein of a non-typeable strain of *Haemophilus influenzae*.

3. The immunogenic composition of claim 2 wherein said HMW protein is a HMW1 or HMW2 protein of the non-typeable strain of *Haemophilus influenzae*.

4. The immunogenic composition of claim 1 wherein another of the antigens which is an adhesin is a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae* or a *Haemophilus influenzae* surface fibril (Hsf) protein of a typeable strain of *Haemophilus influenzae*.

5. The immunogenic composition of claim 1 wherein an antigen of *Haemophilus influenzae* which is not an adhesin is a non-proteolytic heat shock protein of a strain of *Haemophilus influenzae*.

6. The immunogenic composition of claim 5 wherein the non-proteolytic heat shock protein of a strain of *Haemophilus influenzae* is an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease

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activity which is less than about 10% of that of natural Hin47 protein.

7. The immunogenic composition of claim 1 wherein one of said antigens which is an adhesin is an outer membrane protein of *Moraxella catarrhalis* having an apparent molecular mass of about 200 kDa, as determined by SDS-PAGE.

8. A multi-valent immunogenic composition for conferring protection in a host against disease caused by both *Haemophilus influenzae* and *Moraxella catarrhalis*, which comprises:

(a) an analog of *Haemophilus influenzae* Hin47 protein having a decreased protease activity which is less than about 10% of natural Hin47 protein,

(b) a *Haemophilus influenzae* adhesin (Hia) protein of a non-typeable strain of *Haemophilus influenzae*,

(c) a high molecular weight (HMW) protein of a strain of non-typeable *Haemophilus influenzae*, and

(d) an outer membrane protein of *Moraxella catarrhalis* having an apparent molecular mass of about 200 kDa, as determined by SDS-PAGE.

9. The composition of claim 8 wherein said Hin47, Hia, HMW and 200 kDa proteins are present in amounts which do not impair the individual immunogenicities of the proteins.

10. The composition of claim 9 wherein said analog of Hin47 protein is one in which at least one amino acid of the natural Hin47 protein contributing to protease activity has been deleted or replaced by a different amino acid and which has substantially the same immunogenic properties as natural Hin47 protein.

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11. The composition of claim 10 wherein said at least one amino acid is selected from the group consisting of amino acids 91, 121 and 195 to 201 of natural Hin47 protein.
12. The composition of claim 11 wherein Serine-197 is replaced by alanine.
13. The composition of claim 11 wherein Histidine-91 is replaced by alanine, lysine or arginine.
14. The composition of claim 13 wherein Histidine-91 is replaced alanine.
15. The composition of claim 11 wherein Asp-121 is replaced by alanine.
16. The composition of claim 9 wherein said Hia protein is produced recombinantly.
17. The composition of claim 16 wherein said recombinantly-produced Hia protein is an N-terminal truncation V38 rHia.
18. The composition of claim 9 wherein said HMW protein is an HMW1 or HMW2 protein of a non-typeable strain of *Haemophilus influenzae*.
19. The composition of claim 18 wherein the HMW1 and HMW2 proteins are produced recombinantly.
20. The composition of claim 19 wherein said HMW1 and HMW2 proteins are derived from the respective strain of non-typeable *Haemophilus influenzae* and possess respective molecular weights as set forth in the following Table:

Molecular Weight (kDa) non-typeable *H. influenzae* Strain

	12	JoyC	K21	LCDC2	PMH1	15
Mature Protein: HMW1	125	125.9	104.4	114.0	102.4	103.5
HMW2	120	100.9		111.7	103.9	121.9

21. The composition of claim 9 wherein said 200 kDa protein is produced recombinantly.
22. The composition of claim 21 wherein recombinantly-produced 200 kDa protein is an N-terminal truncation V56 r200 kDa.
23. The composition of claim 8 further comprising an adjuvant.
24. The composition of claim 23 wherein said adjuvant is aluminum hydroxide or aluminum phosphate.
25. The composition of claim 8 comprising
- (a) about 25 to about 100  $\mu$ g of the Hin47 protein analog, and
  - (b) about 25 to about 100  $\mu$ g of the Hia protein,
  - (c) about 25 to about 100  $\mu$ g of the HMW protein, and
  - (d) about 25 to about 100  $\mu$ g of the 200 kDa protein.
26. The composition of claim 8 further comprising at least one additional antigenic component for conferring protection against infection caused by another pathogen.
27. The composition of claim 8 wherein said at least one additional antigenic component is selected from the group consisting of diphtheria toxoid, tetanus toxoid, pertussis antigens, non-virulent poliovirus and PRP-T.
28. The composition of claim 27 wherein said pertussis antigens are selected from the group consisting of pertussis toxoid, filamentous hemagglutinin, pertactin and agglutinogens.
29. A method of immunizing a host against disease caused by infection with both *Haemophilus influenzae* and *Moraxella catarrhalis*, including otitis media, which comprises administering to the host an

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immunoeffective amount of a composition as claimed in  
claim 1 or 8.

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ABSTRACT OF THE DISCLOSURE

A multi-valent immunogenic composition confers protection on an immunized host against infection caused by both *Haemophilus influenzae* and *Moraxella catarrhalis*. Such composition comprises at least four antigens comprising at least one antigen from *Haemophilus influenzae*, and at least one antigen from *Moraxella catarrhalis*. Three of the antigens are adhesins. High molecular weight (HMW) proteins and *Haemophilus influenzae* adhesin (Hia) proteins of non-typeable *Haemophilus* and a 200 kDa outer membrane protein of *Moraxella catarrhalis* comprise the adhesin components while the other antigen is a non-proteolytic analog of Hin47 protein. Each component does not impair the immunogenicity of the others. The multi-valent immunogenic composition may be combined with DTP component vaccines, which may also include non-virulent poliovirus and PRP-T, to provide a component vaccine without impairment of the immunogenic properties of the other antigens.

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# Anti-H91A Antibody Titers in Mice

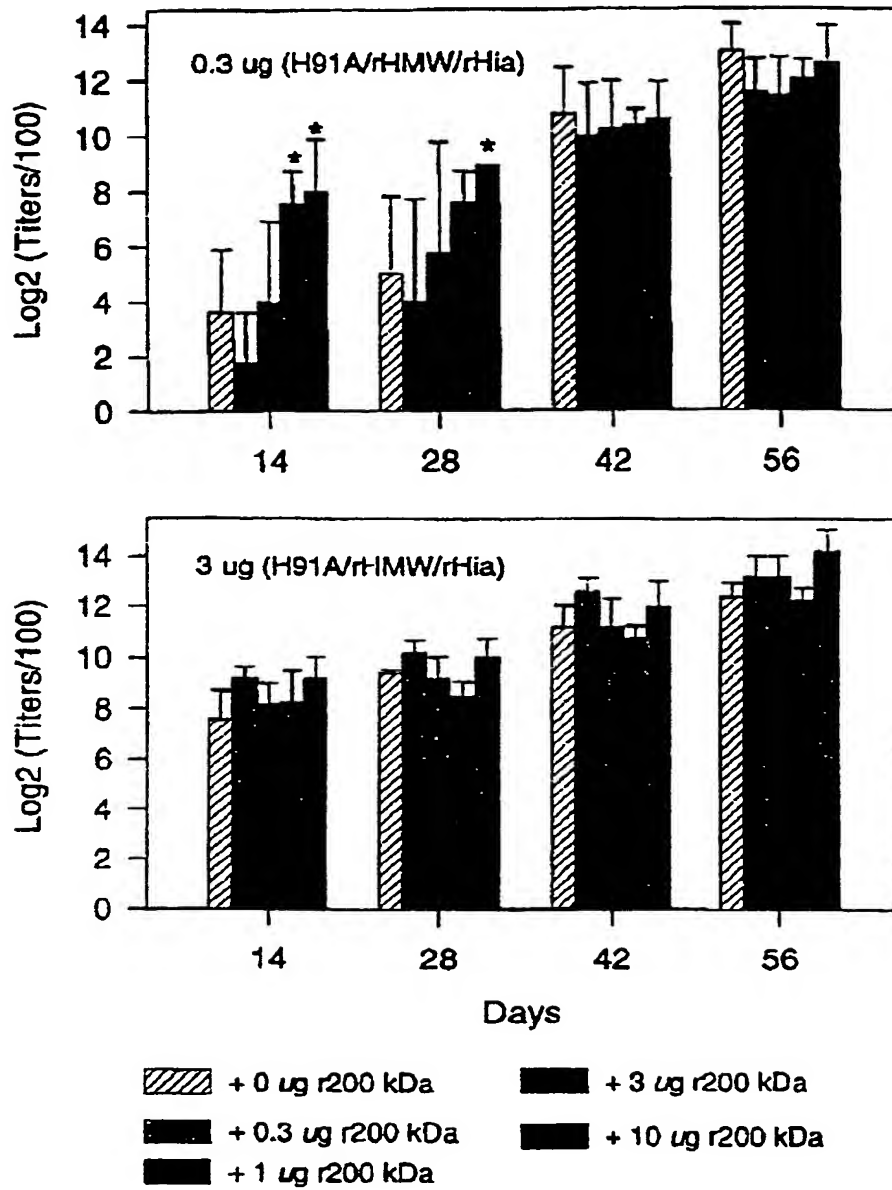


Fig. 1

# Anti-rHMW Antibody Titers in Mice

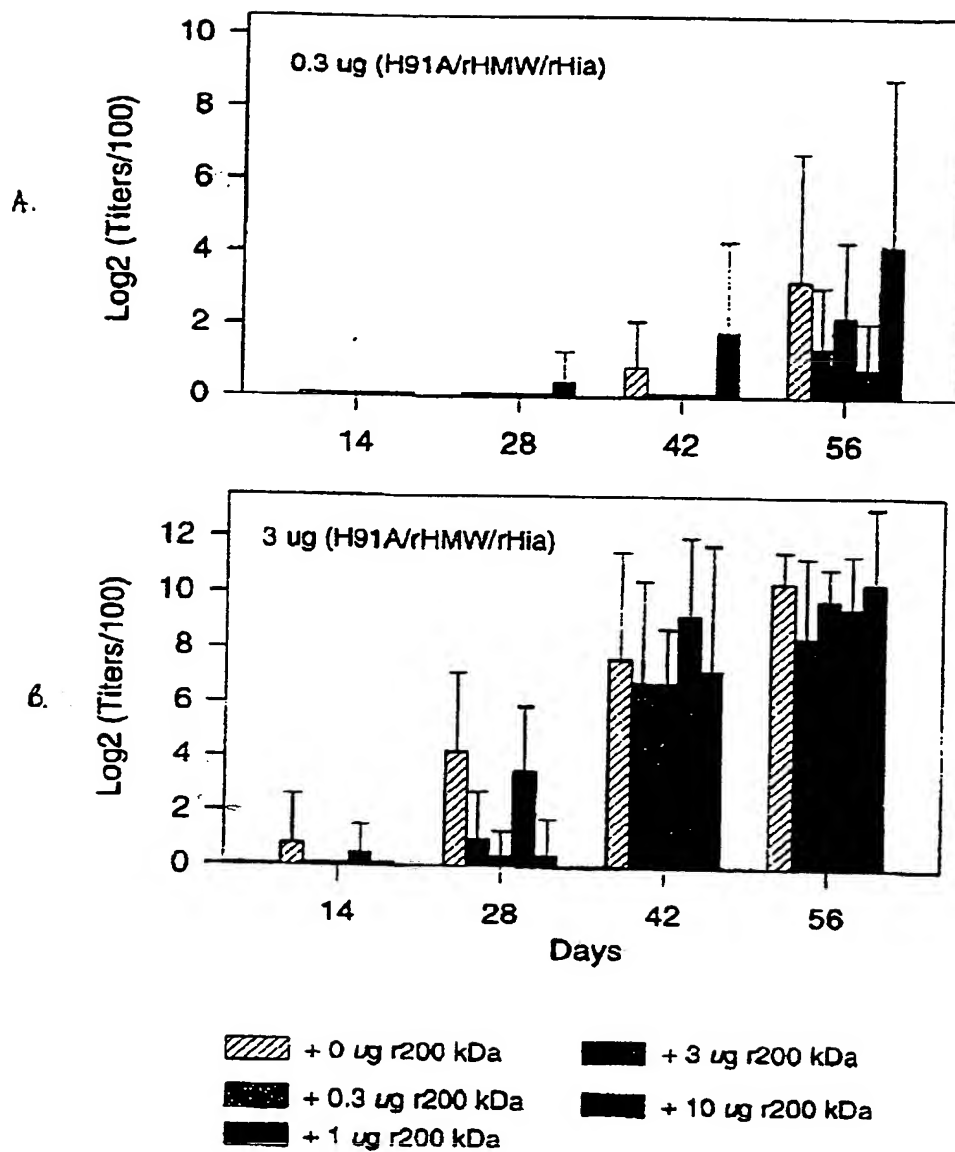


Fig. 2

# Anti-rHia Antibody Titers in Mice

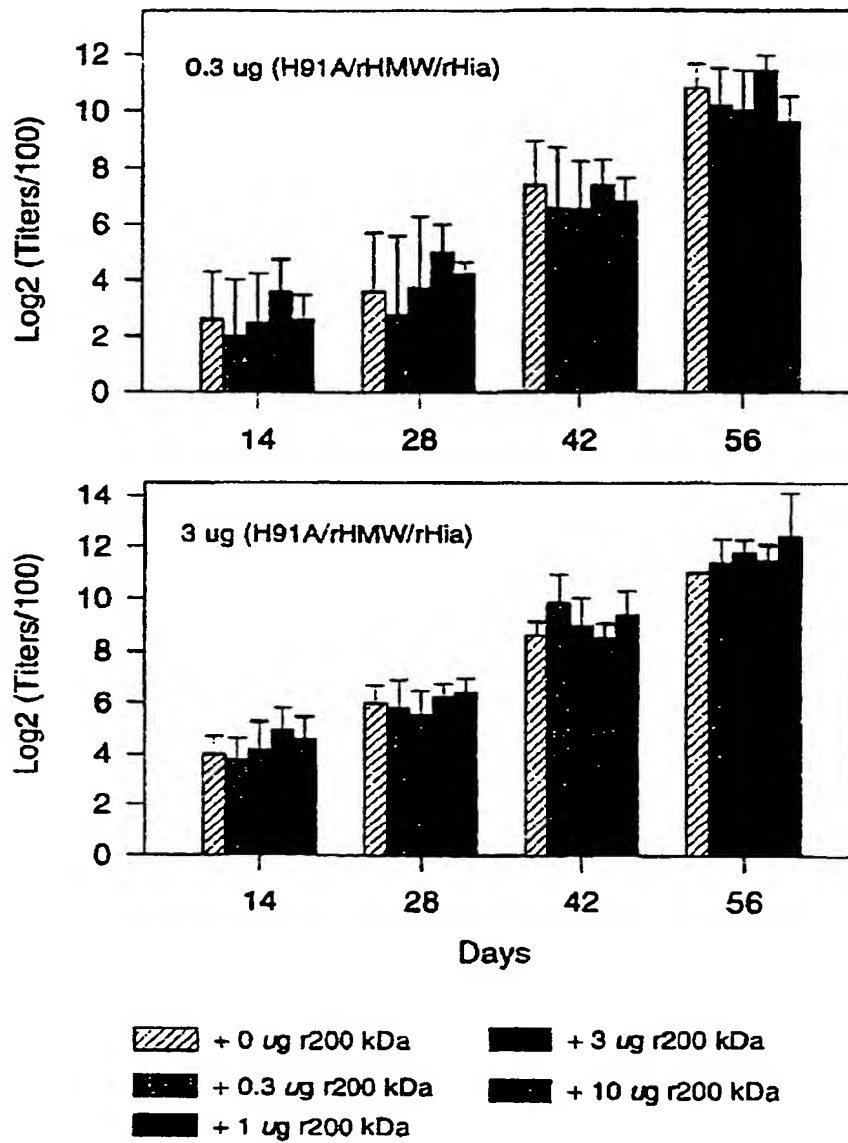


Fig. 3

# Anti-r200 kDa Antibody Titters in Mice

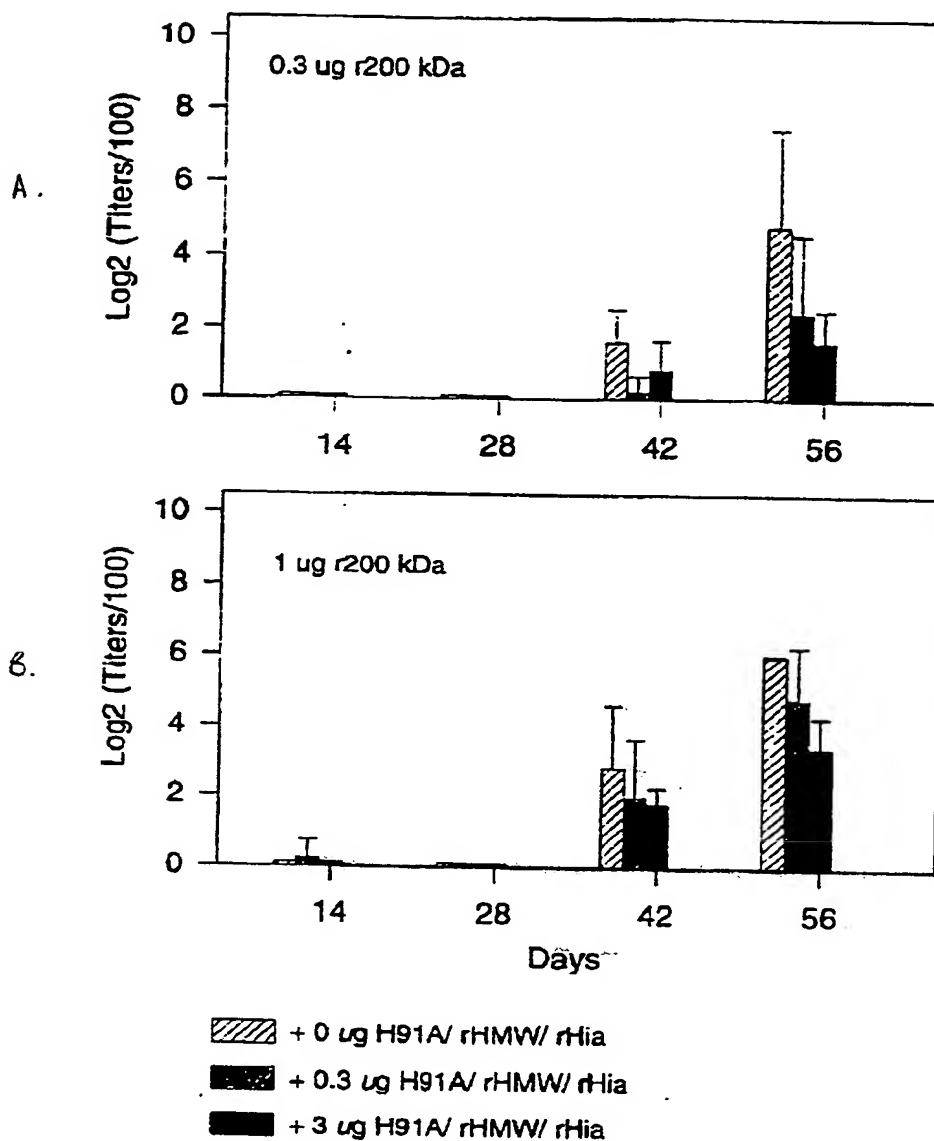


Fig. 4

# Anti-r200 kDa Antibody Titers in Mice

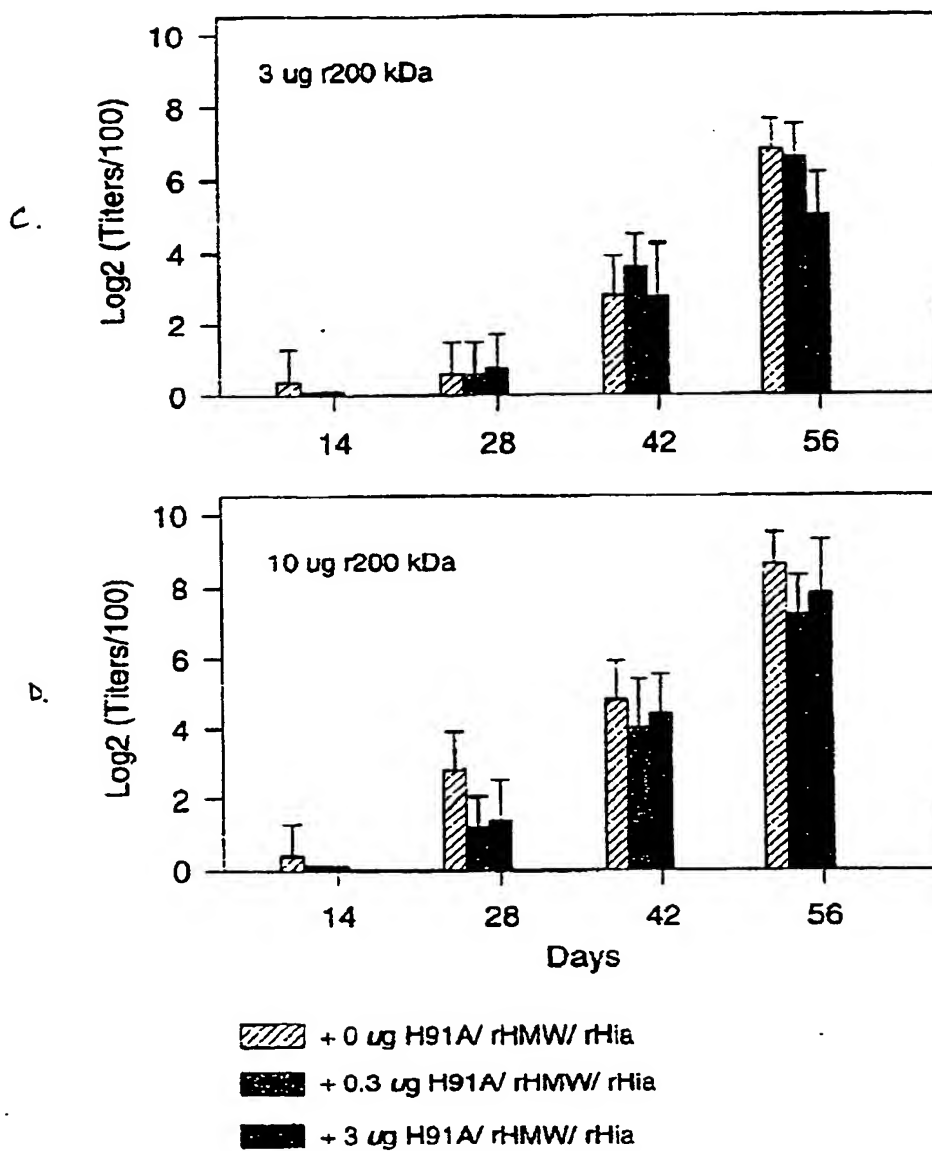


Fig. 4

# Anti-H91AHin47 Antibody Titers in Guinea Pigs

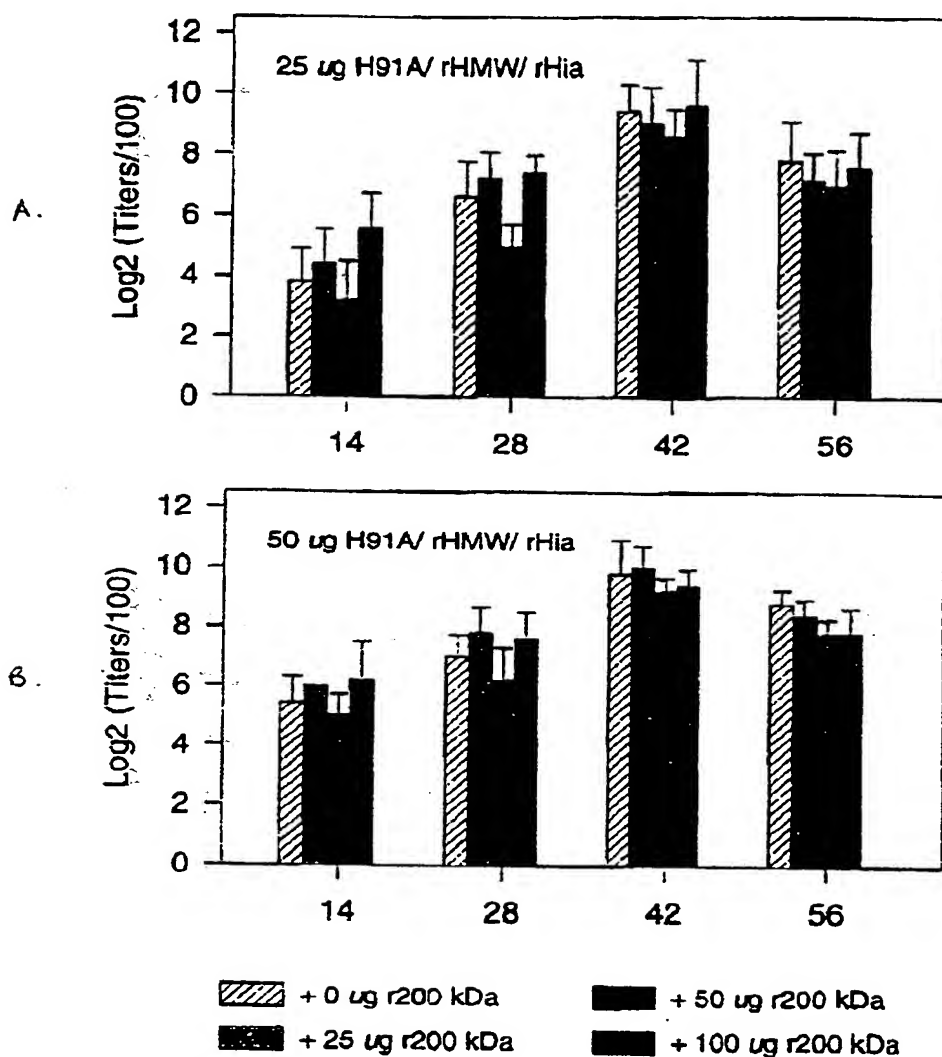


Fig. 5

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# Anti-rHMW1 Antibody Titers in Guinea Pigs

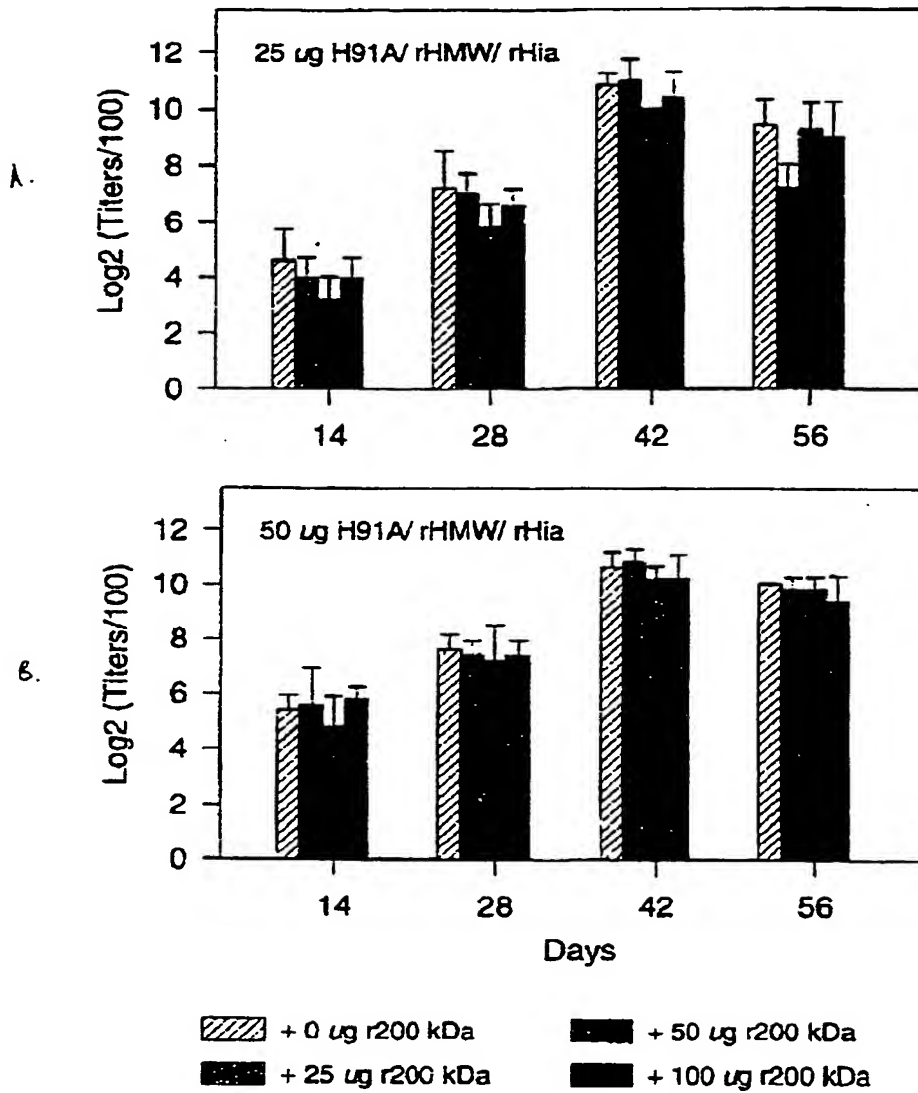


Fig. 6

# Anti-rHia Antibody Titers in Guinea Pigs

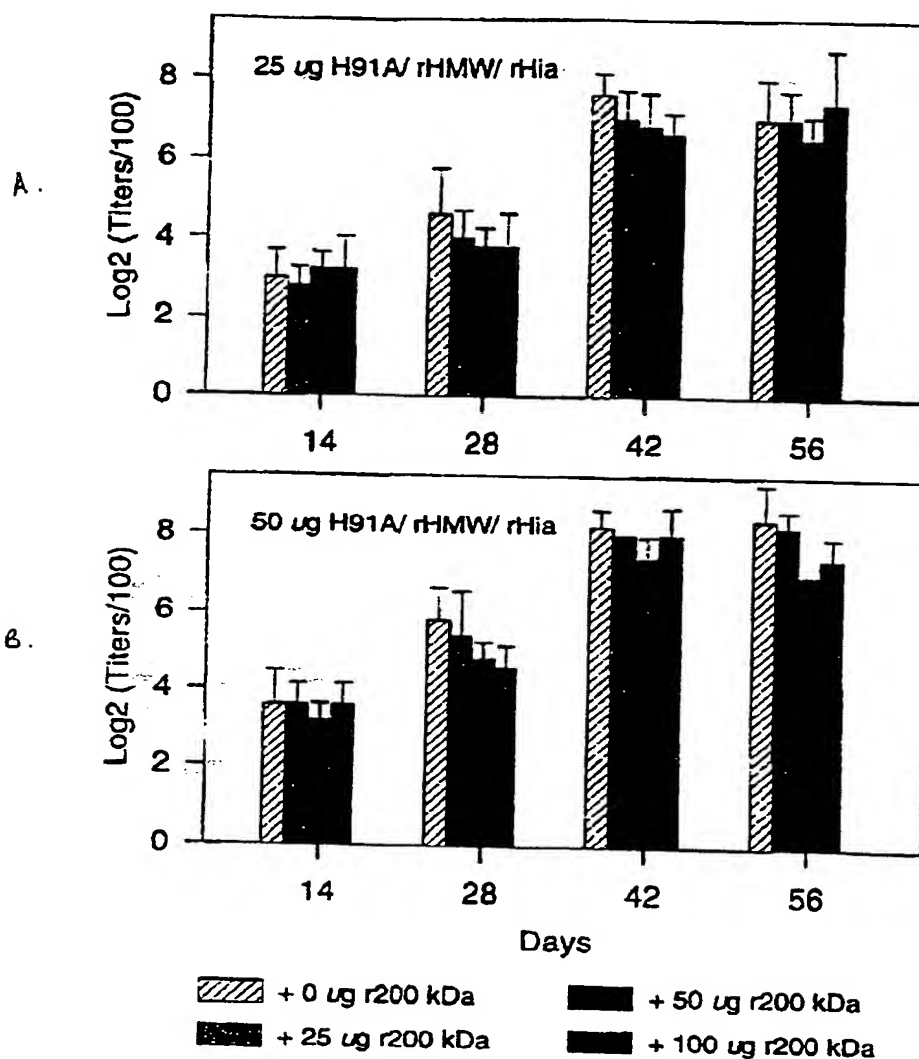


Fig. 7



## Anti-r200 kDa Antibody Titers in Guinea Pigs

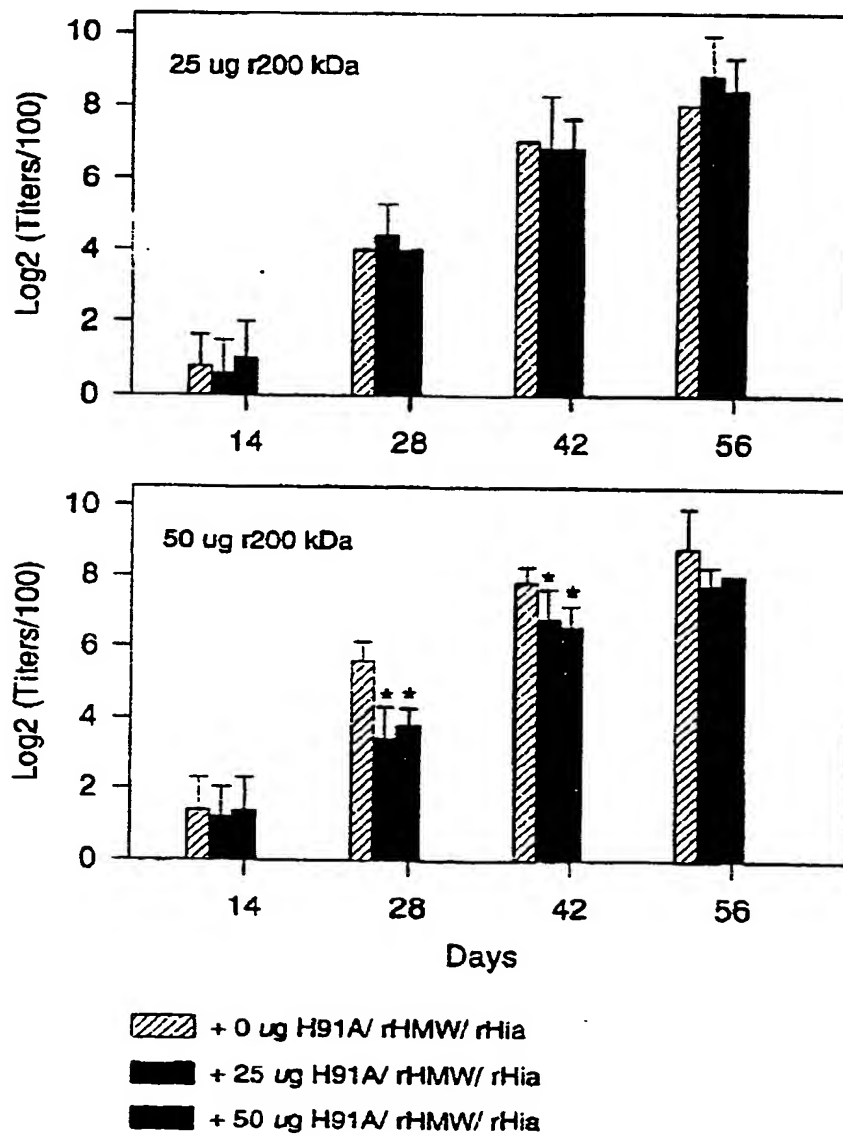


Fig. 8.

# Anti-r200 kDa Antibody Titers in Guinea Pigs

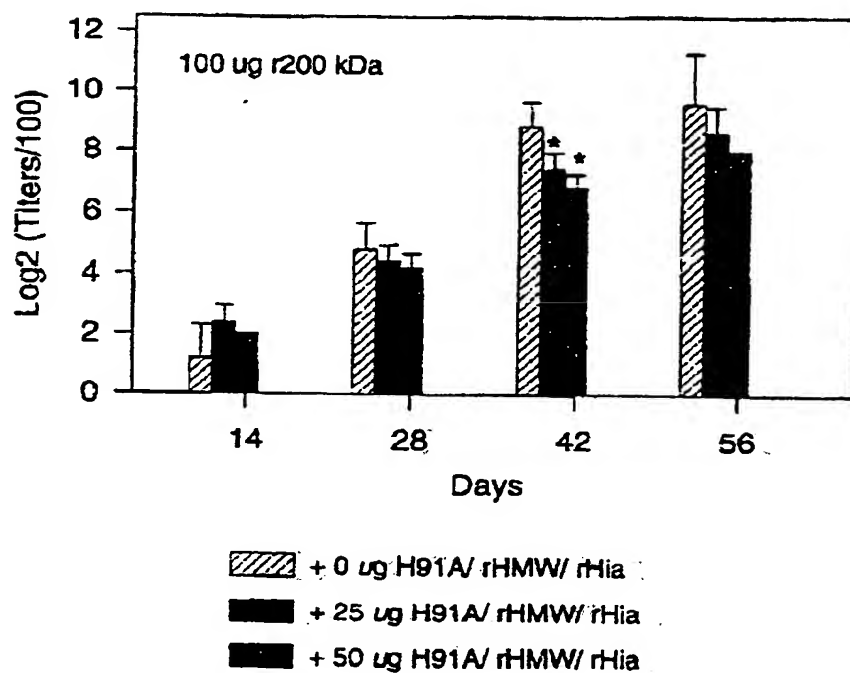


Fig. 8

665720 2195660

FOUR COMPONENT VACCINE

# Protective Ability of rHMW1 Against NP Colonization with NTHi Strain 12 in Chinchillas

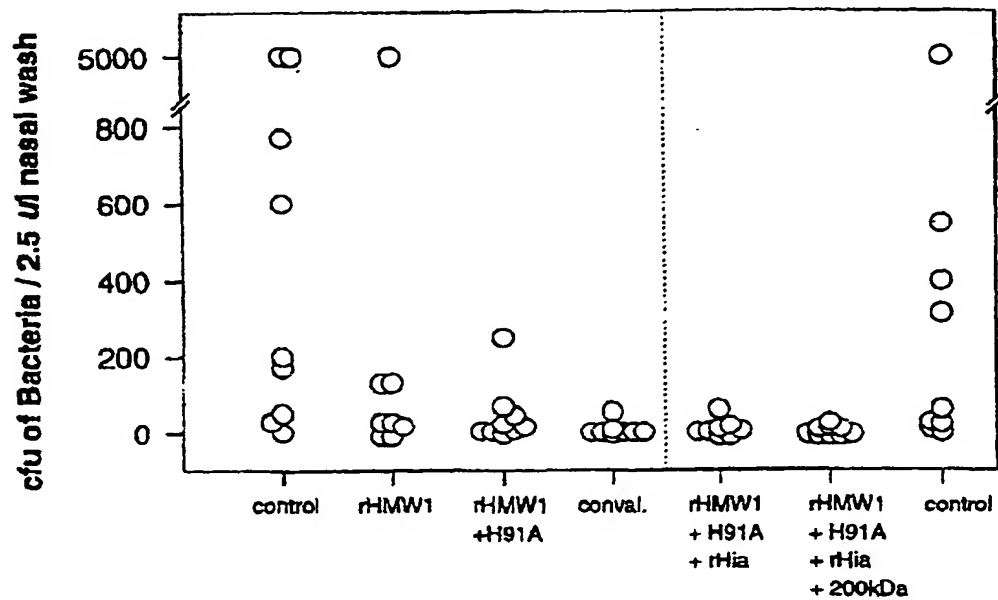


Fig. 9

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*four component vaccine*  
**Protective Ability of H91A Against Intrabulla Challenge  
with NTHi Strain 12 In Chinchillas**

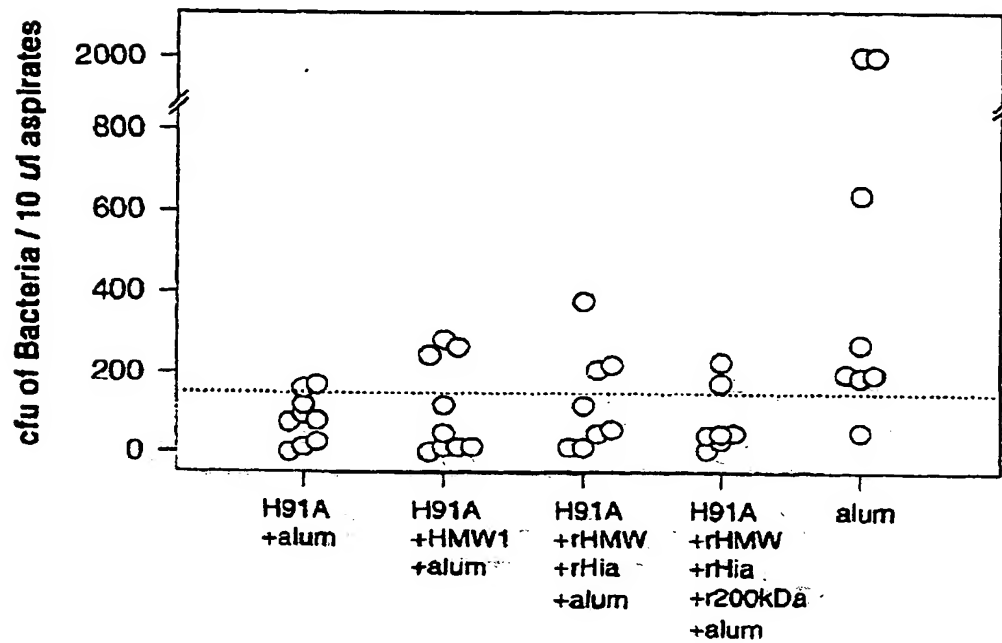
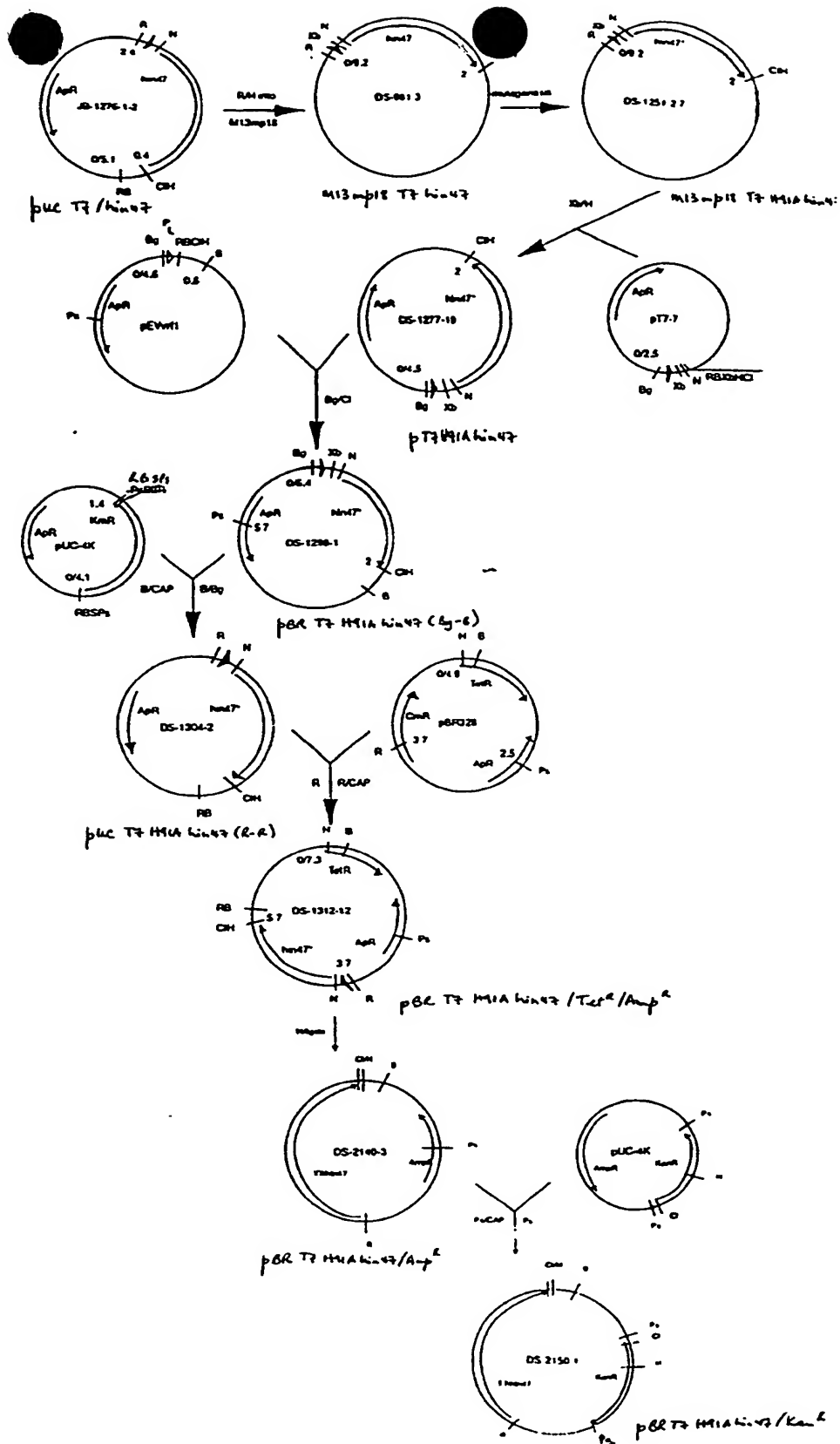
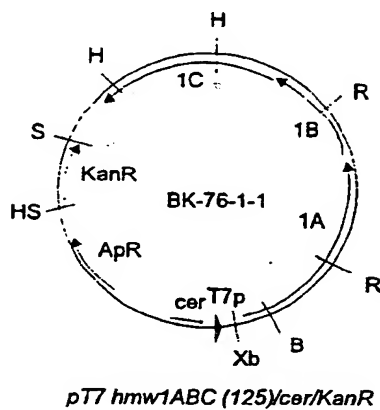
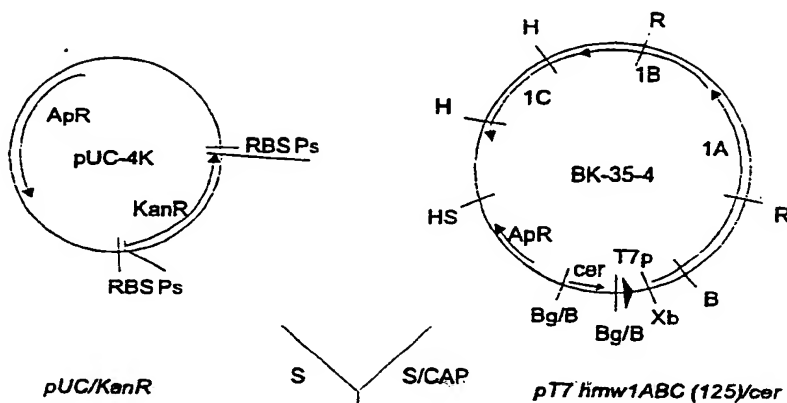
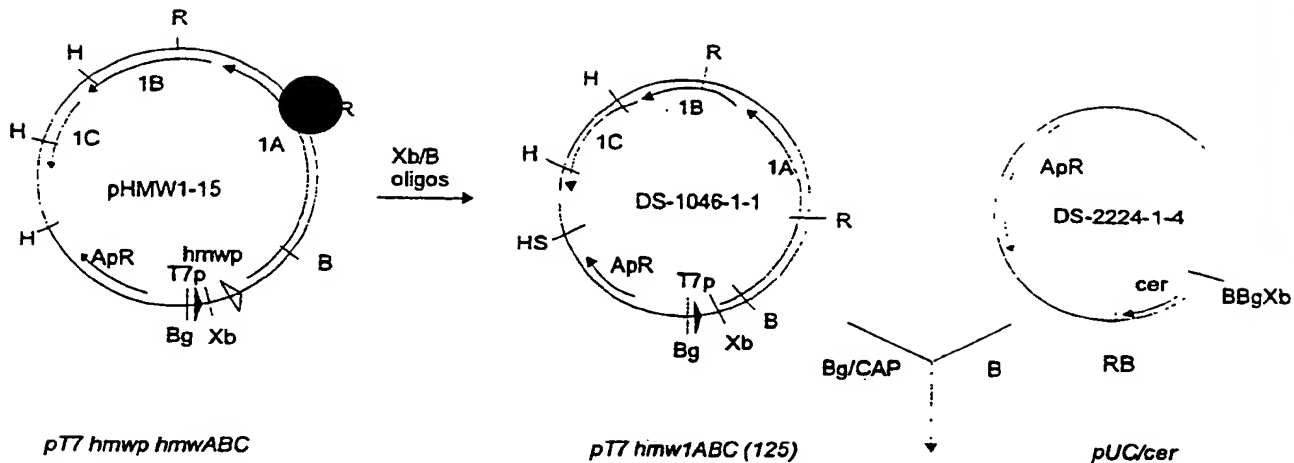


Fig. 10

FIG. 11



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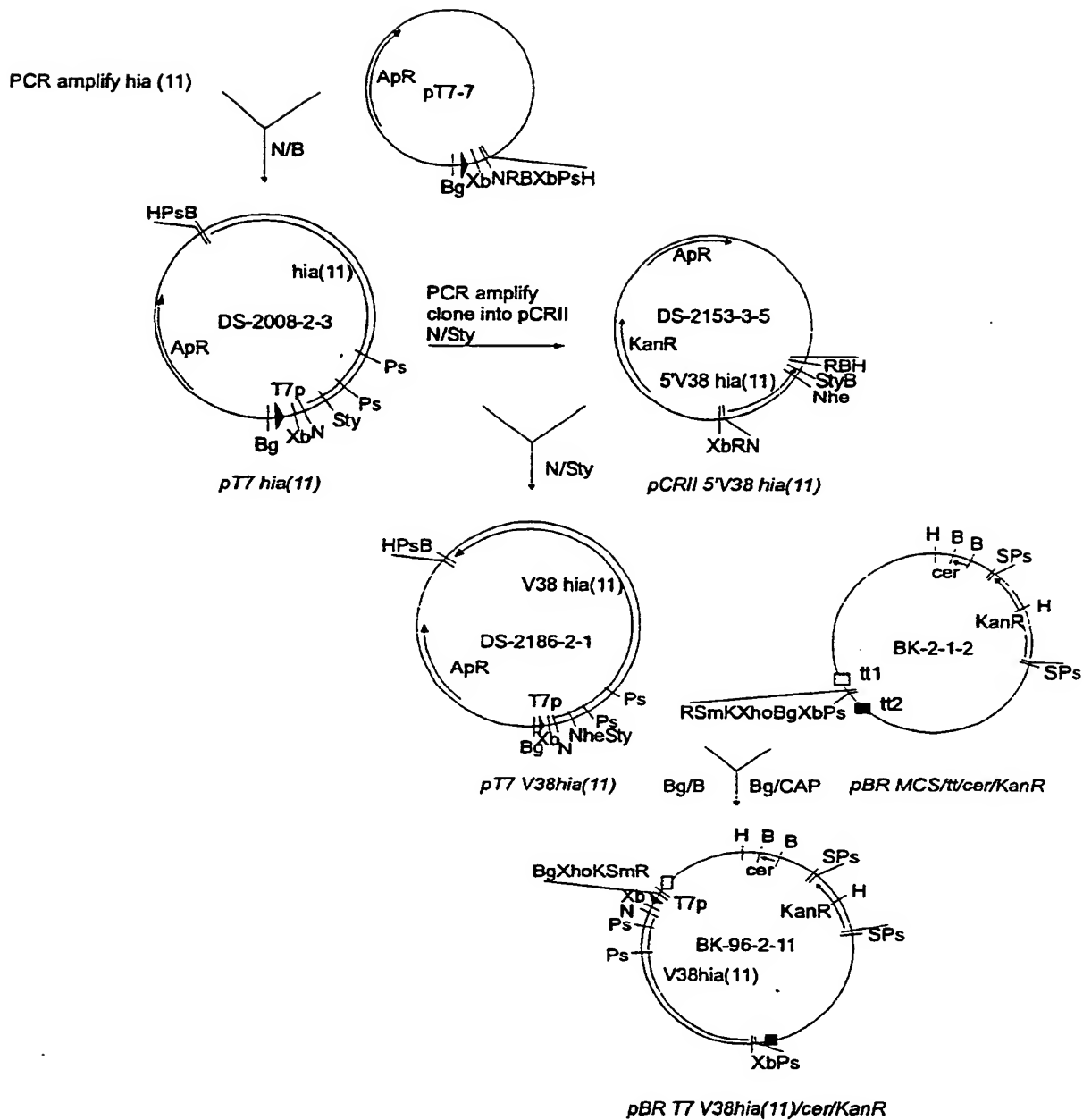
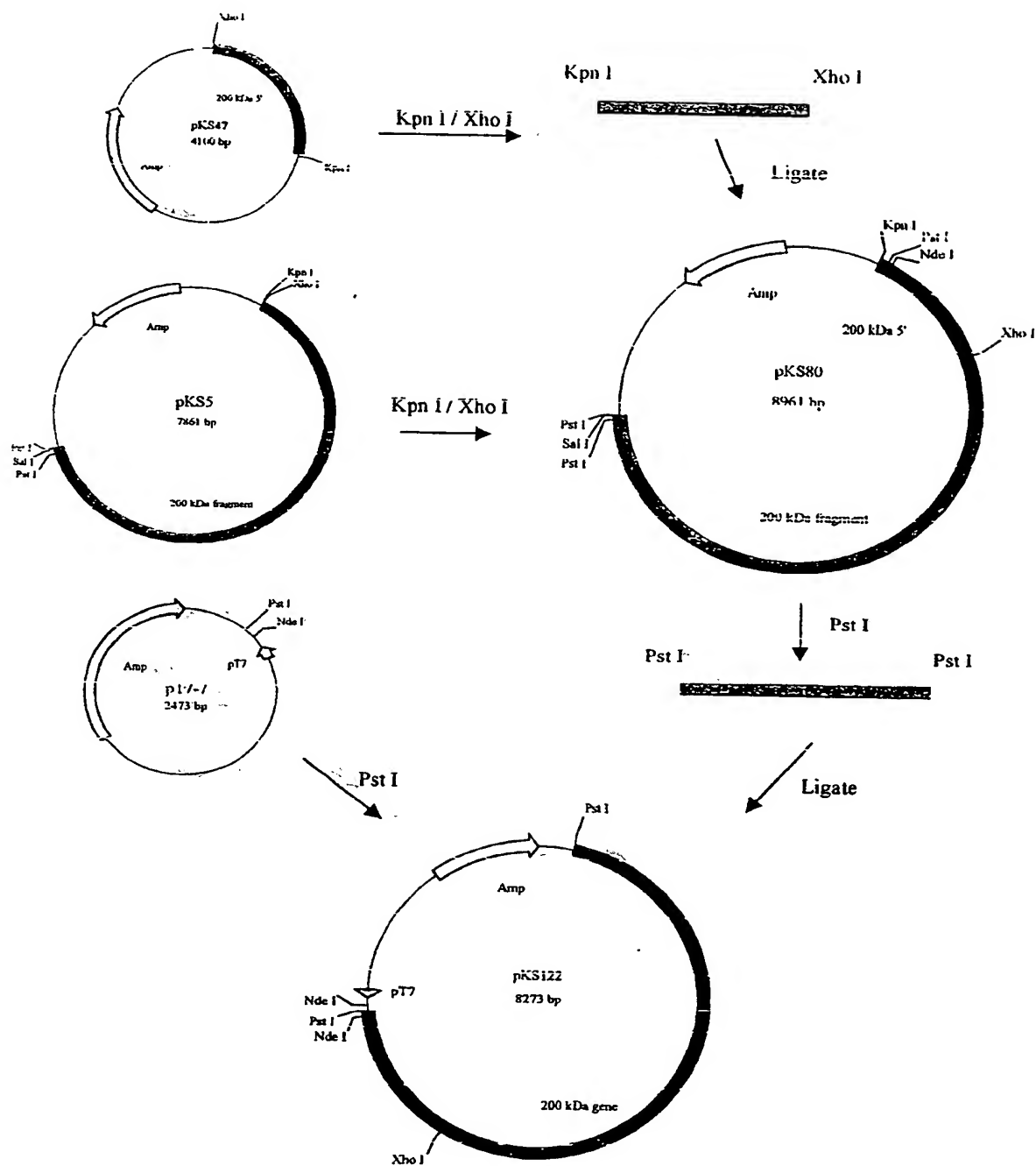


Fig. 13

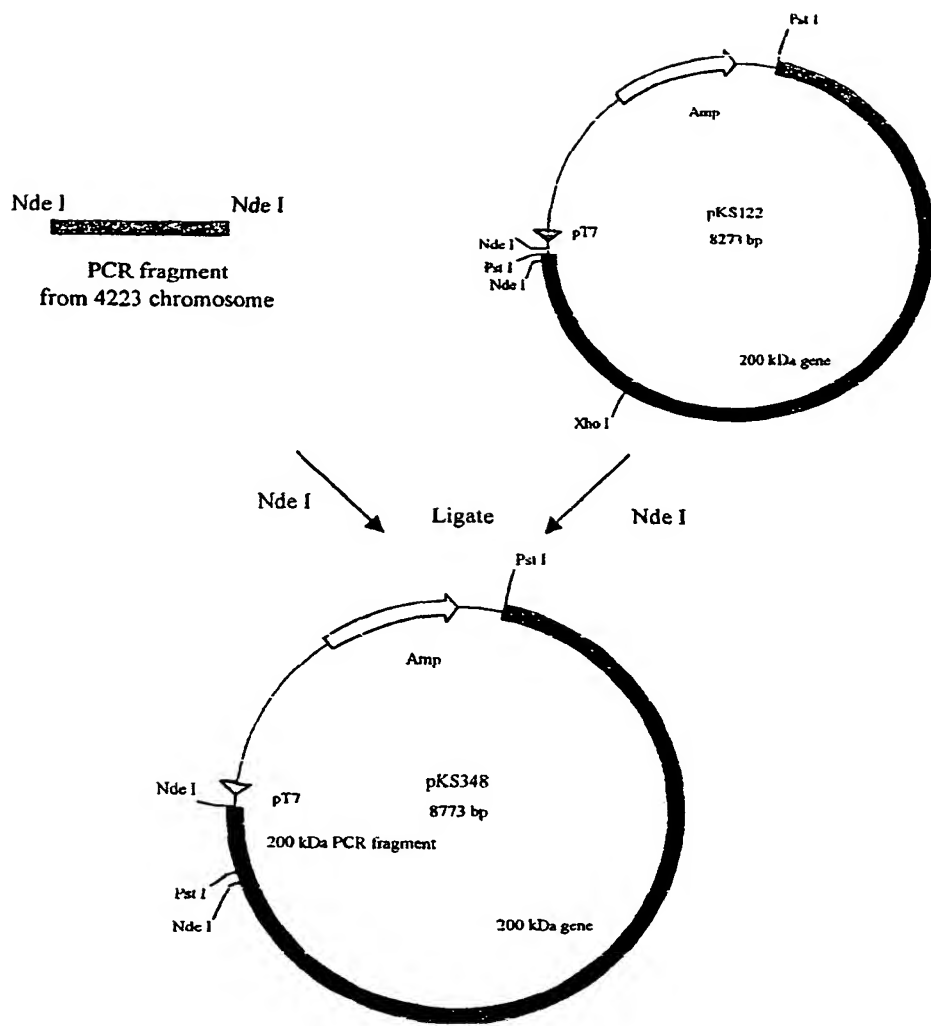
144  
Figure 9A Construction of pKS204 349



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148  
 Figure 14 Construction of pKS348



093637.0150  
 665740 2792560

Docket No.  
1038-868 MIS:jb

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**MULTI-COMPONENT VACCINE TO PROTECT AGAINST DISEASE CAUSED BY HAEMOPHILUS INFLUENZAE AND MORAXELLA CATARRHALIS**

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as United States Application No. or PCT International Application Number \_\_\_\_\_ and was amended on \_\_\_\_\_

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

(Number)

(Country)

(Day/Month/Year Filed)

☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

_____ (Application Serial No.)	_____ (Filing Date)
_____ (Application Serial No.)	_____ (Filing Date)
_____ (Application Serial No.)	_____ (Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)
_____ (Application Serial No.)	_____ (Filing Date)	_____ (Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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Fourth inventor's signature	Date
Residence	
Citizenship	
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Full name of fifth inventor, if any	
Fifth inventor's signature	Date
Residence	
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Post Office Address	

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Sixth inventor's signature	Date
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Full name of sixth inventor, if any	
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Citizenship	
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